Comparative studies on O-methyltransferases involved in lignin biosynthesis(Dissertation_全文)

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COMPARATIVE STUDIES ON O-METHYLTRANSFERASES INVOLVED IN LIGNIN BIOSYNTHESIS

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1981

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INTRODUCTION

INTRODUCTION

A FOREST TREE is often compared to a building which is made of reinforced concrete. Just like concrete in the building, lignin (reviews cited in reference 1-5) plays a role to fix cellulose and hemicelluloses in plant cell walls, supporting trees against intensive stress. The lignins can be readily classified by investigating the degradation products and by the color reaction such as Mäule and Cl_2-Na_2SO_3 reactions.[6] Comparative studies of lignin structural units have provided an interesting phylogenic difference in plant kingdom.[7-11] The lower land plants such as club moss, fern and gymnosperms contain guaiacyl lignins whereas plants of more recent origin such as angiosperms, contain guaiacyl-syringyl lignins. Additional lignin stuructural unit, p-hydroxyphenylpropane is found in Gramineae lignins, although there are some exceptions to these generalization, which are shown in Table 1.

ble 1
(p. 11)

Lignin is widely distributed as a cell wall constituent in terrestrial vascular plants, except Tracheophyta plants grown in buoyant aqueous solution. Bacteria, fungi, red and green algae contain no flavonoids[12] and lignins. On the other hand, mosses and liverworts generally contain a few flavonoids[12] but no lignin. Most of the aquatic angiosperms which degenerate their vessel elements, contain a trace amount of (or no) lignin. It may be ascribed to that the plants need

neither the accumulation of waste products nor strengthen the mechanical elements with lignin because of the aquatic life type. Vessel elements[13-15] are widely distributed in angiosperm. Exceptional angiosperms which lack vessel elements are concentrated in Polycarpiidae in addition to the aquatic plants described above. Polycarpiidae is considered to be one of the most primitive groups in angiosperms,[16] the lignin of which demonstrated to contain syringyl units but Sarcandra one. On the other hand, the vessel elements are observed in several genera of Pteridophyta and Gymnospermae, the lignins of which are known to contain syringyl units in some species. These facts indicate that the presence of vessel elements is not directly related to the phylogenic distribution of syringyl units. These are summarized in Table 1 and 2.

ble 2
(P. 12)

Most of the lignin biosynthetic pathway was elucidated by tracer experiments in nineteen fifty's and sixty's, although the pathway was mainly studied in grass plants.[1-3] During the last decade, attentions are forcused on the cell free system in lignin biosynthetic pathway and the regulatory mechanisms in the biosynthesis have been investigated.[17-27] Therefore, following paragraphs outline some important enzymes which is participating in lignin biosynthesis.

Since phenylalanine ammonia-lyase (EC 4.3.1.5; PAL) was first extracted from Hordeum vulgare,[28] many important aspects

have been elucidated and reviewed in this enzyme.[25,29,30] The enzyme catalyzes the elimination of the pro-S proton from C-3 of L-phenylalanine in an antiperiplanar fashion to form trans-cinnamate, together with ammonia. The reaction mechanism of this enzyme may be explained by Ordered Uni Bi sequence proposed by Havir and Hanson.[31] Km values for the substrate fall in the range from 10^{-5} to 10^{-2} M. The enzymes are strongly inhibited by cinnamate, and some of them were inhibited by phenolics as well; one of the PALs in Ipomoea batatas is sensitive to p-coumarate and caffeate in addition to the above compounds.[32] Such an example was also found in differnt cell organelles in Quercus.[33] PALs from the microsomal and 10^{4}G precipitated fractions in the Quercus plant were suggested to be involved in C_6-C_3 and C_6-C_1 metabolisms, respectively, because the former was inhibited by cinnamate and the latter by vanillate. PAL has been so far found in all green plants including Pteridophyta,[34] but is not widely distributed in mosses. No PAL activity was detected in lichens and fungi with the exception in certain higher Basidiomycetes,[35,3] and Streptomyces.[37,38] PAL in algae is only reported in Porphyridium.[30] Thus, the phylogenic distribution of this enyzme is closely related to the phylogenic occurrence of lignins.

Cinnamate 4-hydroxylase (EC 1.14.13.11)[25] was characterized as a microsomal monooxygenase which catalyzes cinnamate 4-

hydroxylation with NIH shift.[39] The three substrates, i.e. cinnamate, NADPH and O_2 showed Km values in micromolar ranges, and the reaction was inhibited by p-coumarate. This enzyme may require P450 as an electron acceptor because the enzyme in Sorghum is inhibited by carbon monooxide and the inhibition was recovered by light.[40]

Phenolase (EC 1.10.3.1) functions as an oxygenase (EC 1.14.18.1) which catalyzes the hydroxylation of p-coumarate to caffeate with electron donors such as ascorbate, NAD(P)H, tetrahydropteridine derivatives and o-diphenols.[19,25] It is proposed that $\cdot O_2^-$ generated by illuminated chloroplasts is involved in the hydroxylation.[45] The phenolase, which is a copper enzyme and is inhibited by carbon monooxide and not recovered by light, localized in both soluble and bound forms. The metabolic intermediates such as cinnamate, benzoate and ferulate are found to be potent inhibitors of this enzyme.[17] Another possible reaction of this step may be a peroxidase-mediating reaction,[41] although this reaction is known to occur non-enzymatically. It is noteworthy that the mixed function enzymes in Sorghum alter the raios of oxygenase to oxidase with their molecular weights[42] and that the interconversible subunit structure was reported in the phenolase.[43] Another interesting phenolase which can convert p-coumarate to caffeate was reported to be present in a microsomal fraction in Quercus. However, ferulate-5-hydroxylase is still unsuccessful to iso-

late in cell free. A series of enzymes which catalyze the formation of C_6-C_3 phenolic acids from phenylalanine might be expected to exist as a multiple enzyme complex.[19]

Cinnamate derivatives are activated to the corresponding esters by cinnamate:CoA ligase (EC 6.2.1.12) prior to the lignin, flavonoid and ester formations.[26] The enzyme catalyzes the formation of cinnamoyl-CoA derivatives from the corresponding acids, with ATP, CoA and Mg^{2+}. The ligase is inhibited by cinnamate, CoA and ATP,[47,48] which are the substrates and a product, respectively. The ligase isoenzymes were found in soybean (*Glycine max*) cultured cells,[48] petunia (*Petunia hybrida*)[49] and other tissues.[27,50] One of them catalyzes the esterification of *p*-coumarate, feruralte, 5-hydroxyferulate and sinapate, while the other catalyzes the same esterification except sinapoyl-CoA formation in the soybean cultured cells. Hahlbrock and Grisebach assumed that the former isoenzyme is involved in lignin and the latter in flavonoid biosynthesis, respectively, although the evidence was insufficient.[24] On the other hand, Gross[27] and Kutsuki *et al.*[51] pointed out that the former isoenzyme is not universally distributed even in angiosperm, although the gymnosperm enzyme was assumed to be the latter type. This is an interesting finding because lignification is a common phenomenon in the angiosperm. The details in this point will be discussed in chapter II and IV.

The cinnamoyl-CoA derivatives are reduced to the correspond-

ing alcohols in lignin biosynthesis,[47] the reaction of which are catalyzed by cinnamoyl-CoA: NADPH oxidoreductase (EC 1.1.1.-)[52,54] and cinnamyl alcohol: NADP oxidoreductase (EC 1.1.1.-).[53,55] The former and the latter enzymes belong to B-[52] and A-groups[53] of NAD(P)-specific dehydrogenase, respectively. The best substrate for the former enzyme was feruloyl-CoA among the cinnamoyl-CoA derivatives in soybean cultured cells[54] and Forsythia young shoots.[52] The reaction products, CoA and $NADP^+$, inhibited the reduction, and the inhibition kinetics indicated a random reaction.[54] The phylogenic distribution of this enzyme in gymnosperm and angiosperm is not reported yet, because of the instability of the enzyme. In the second reduction step from the cinnamaldehydes to the corresponding alcohols, the enzyme was strongyl inhibited by the cinnamyl aldehydes which served as substrates.[55] The two isoenzymes were reported in soybean cultured cells.[55] One of them catalyzes only coniferyl alcohol formation and the other catalyzes both coniferyl and sinapyl alcohol formations. Gymnosperm only contains former type enzyme.[51]

Peroxidase (EC 1.11.1.7) was classified into land type and marine type.[18] The former enzyme is distributed in green algae and the terrestrial vascular plants including aquatic angiosperm, which catalyzes the oxidation of guaiacyl compounds. On the other hand, the latter enzyme which is distributed in red and brown algae, is not able to catalyze the oxidation of

guaiacyl compounds. Polymorphism of this enzyme is well known and the each multiple form showed different substrate specificities in guaiacyl and syringyl compounds.[56] Another interesting aspect of the enzyme is the finding that peroxidase produces hydrogen peroxide with malate dehydrogenase coupling system with oxygen.[57]

The methoxyl content is often examined as an essential criterion to characterize lignins. The methoxyl groups are derived from the methyl group of S-adenosyl-L-methionine, which is a substrate in transmethylation catalyzed by O-methyltransferase (EC 2.1.1.-; OMT). Several plant OMTs participating in various biosynthetic pathway have been characterized since the OMTs in apple (Malus sp.) and Nerine bowdenii were extracted.[58-60] The reported enzymes will be described in the following chapters. Shimada et al. first pointed out that the OMT determines the destiny if lignins carry guaiacyl nuclei or guaiacyl-syringyl ones.[61] They used the ratio of sinapate (SA)- to ferulate (FA)-formation (SA/FA ratio) by OMTs in order to estimate the substrate specificities.

In these circumstances, this thesis forcuses on the OMTs which are participating in lignin biosynthesis in taxonomically different plants and fills the ambiguous parts in the

enzyme properties.

In chapter I, pine (Pinus thunbergii) OMT is described in details as a typical gymnosperm-type enzyme. Gymnosperm enzymes had never been purified until this enzyme was isolated from the pine seedlings and characterized.

Aspen (Populus euramericana) OMT is discussed as a typical dicotyledon-type enzyme in chapter II. It is desirable that secondary xylem in woody plants is used for studying lignin biosynthesis. However, heavily lignified tissues are usually unsuitable as enzyme sources because of its toughness. The OMT was extracted from the tree trunks, which was an attempt to characterize enzymes from such heavily lignified tissues.

Mistletoe (Viscum album) lignin was a typical angiosperm type as it is demonstrated in chapter III. Mistletoe OMT is discussed in this chapter, because the crude enzyme was found to show apparently no ferulate formation which may remind us of heterotrophism of the parasitic plant. The unusual substrate specificity is discussed in relation to the question if the parasitic plant has a lignin biosynthetic pathway.

In chapter IV, bamboo (Phyllostacys pubescens) OMT is discussed as a typical grass type enzyme. In this chapter, it is clearly demonstrated that the FA- and SA-activities are originated from the same enzyme. This finding indicates

that the ratios of FA- to SA-foramtion keep constant during the differentiation.

In chapter V, the three types of OMT are summarized by estimating SA/FA ratios in various plants and the relationship between OMTs and lignin evolution is discussed.

TABLE 1 EXCEPTIONAL DISTRIBUTION OF SYRINGYL LIGNINS

GENUS OR (FAMILY)	VESSEL ELEMENTS	MÄULE REACTION	SYRING-ALDEHYDE	REMARKS (CLASS)
SELAGINELLA	+	+	+	GLOSSOPSIDA
EQUISETUM	+	−	−	SPHENOPSIDA
PTERIDIUM	+	±		PTEROPSIDA
DENNSTAEDTIA		±		
COLYSIS		+		
ELAPHOGLOSSUM		+		
PLAGIOGYRIA		±		
CERATOPTERIS		+		
ZAMIA		+		CYCADOPSIDA
TETRACLINIS		+	+	CONIFEROPSIDA
PODOCARPUS		±	±	
(WELWITSCHIACEAE)	+	+	+	CHLAMYDOSPERMOPSIDA
(EPHEDRACEAE)	+	+	+	
(GNETACEAE)	+	+	+	
(WINTERACEAE)	−	+	+	ANGIOSPERMOPSIDA
TROCHODENDRON	−	+	+	
TETRACENTRON	−	+	+	
AMBORELLA	−			
SARCANDRA	−	−	+	

TABLE 2 EXCEPTIONAL DISTRIBUTION OF VESSEL ELEMENTS

THE PTERIDOPHYTA AND GYMNOSPERMAE WHICH CARRY VESSEL ELEMENTS	
GLOSSOPSIDA	SELAGINELLA
SPHENOPSIDA	EQUISETALES
PTEROPSIDA	PTERIDIUM
CHLAMYDOSPERMOPSIDA	WELWITSCHIA
	EPHEDRA
	GNETUM
THE ANGIOSPERMAE WHICH LACK VESSEL ELEMENTS	
DICOTYLEDONEAE*	WINTERACEAE
	TROCHODENDRON
	TETRACENTRON
	AMBORELLA
	SARCANDRA
	NYMPHAEACEAE
THE ANGIOSPERMAE WHICH DEGENERATE VESSEL ELEMENTS	
DICOTYLEDONEAE	MYRIOPHYLLUM
	PODOSTEMONACEAE
	MONOTROPASTRUM
MONOCOTYLEDONEAE	HYDROCHARITACEAE
	LEMNACEAE
	NAJADACEAE
	RUPPIACEAE
	ZANNICHELLIACEAE
	ZOSTERACEAE

* ALL THESE PLANTS BELONG TO POLYCARPIIDAE.
THE SCIENTIFIC NAMES ARE ESSENTIALLY BASED ON ref. 145 & 146.

Chapter I Roles of Pine O-Methyltransferase in Gymnosperm Lignin.

INTRODUCTION

Conifer lignins are known to be mainly composed of guaiacyl units, and usually lack syringyl ones.[7,8,11,62-64] A few enzymes were reported in relation to lignin biosynthesis in conifers,[65-68] although the enzymes are generally difficult to extract and characterize[66] as it will be discusssed in chapter V. The gymnosperm lignin is produced via reactions mediated by a series of enzymes which lack catalytic ability for syringyl-unit formation. For example, gymnosperm p-coumaroyl-CoA ligase and p-hydroxycinnamyl alcohol dehydrogenase catalyze guaiacyl-unit formation, but scarcely does syringyl-unit formation.[51,69]

Finding that O-methyltransferase (OMT) catalyzes ferulate (FA)-formation but scarcely does sinapate (SA)-formation, first explained the reason why gymnosperm almost entirely contains guaiacyl lignin.[61] Although a little amount of syringyl lignin in gymnosperm can be explained by the substrate specificity, an OMT-inhibitor might be present and apparently masking sinapate-formation in the crude preparation. Such possibility, as shown in chapter III, is not negligible when the gymnosperm OMT is investigated without purification.

This is the first report on gymnosperm enzymes which were purified and characterized.[67] This chapter describes gymnosperm OMTs, especially purified OMT in Pinus thunbergii, and will be discussed the role of the enzyme in relation to the lignin biosynthesis.

RESULTS

Purification of the Pine OMT in the Seedlings

Pine (Pinus thunbergii) OMT was purified about 90-fold in a yield of 22% with respect to the starting crude homogenate (Table 3). FA- and SA-activities were not resolved by the disc electrophoresis of the final preparation, although the SA-activity was much lower than FA-activity as shown in Figures 1 and 2. However, the ratio of SA- to FA-activities (SA/FA ratio) became smaller during the enzyme purification (0.065 to 0.037), suggesting the removal of SA-activity during the procedure. In fact, a minor fraction, which gave a high SA/FA ratio, was detected in the 0-60% ammonium sulfate precipitate (step 2) by polyacrylamide gel electrophoresis (Table 4) and by gel filtration chromatography on Sephadex G100. This minor fraction was more negatively charged on the gel in the electrophoresis (Figure 3) and eluted faster than the major one in the gel filtration chromatography (Figure 4). The overall SA/FA ratio at step 2 was calculated to be 0.17, the value of which was derived from the electrophoretic data shown in Table 4. This calculated value was higher than that shown in Table 3. A similar discrepancy between the calculated and found ratios was also observed in the fractions separated by gel filtration chromatography, although the causes were not elucidated. In any event, the changes

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p. 25)

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p. 24)

of the ratio during the purification is at least partially explained by the removal of the minor fraction which catalyzes SA-formation.

Some Properties of the Pine OMT

It was previously reported that the crude pine OMT (ammonium sulfate precipitate) was almost completely inhibited by 5 mM of EDTA, and that Mg^{2+} might stimulate the activity.[65] In contrast with the previous results, Table 5 shows that the finally purified enzyme was not only inhibited but also activated by 0.5 mM EDTA or NaF in the absence of Mg^{2+}. Therefore, Mg^{2+} seems to be no absolute requirment for the enzymic activity, although Mg^{2+}, Ca^{2+}, and Mn^{2+} were useful for keeping high enzyme activity. A similar result was lately reported on Thuja OMT.[68] Such properties of the pine OMT were similar to those of purified catechol-O-methyltransferase from human liver, the enzyme of which was stabilized by 0.2 mM $MgCl_2$ and EDTA.[70]

ble 5 (p. 27)

Addition of glycerol was found to be effective against denaturation of the OMT during freeze-thawing, and the presence of 10 mM of iso-ascorbate, cystein and 2-mercaptoethanol was necessary for the full enzymic methylation. The provisional molecular weight of the major fraction at step 2 was estimated to be 6.7×10^4 by the gel filtration on Sephadex G100 (Figure 4), the value of which is comparable to that of Thuja OMT.

g. 4 (p. 26)

Kinetic Studies on the Pine OMT

Km value for caffeate was 51.1 μM which is hundred times lower than that of parsley OMT.[71] The difference seems to be ascribed to the fact that the parsley OMT is a flavonoid specific one whereas the pine OMT is only responsible for the biosynthesis of guaiacyl lignin. The kinetic constants summarized in Table 6 show that the affinity of the enzyme for caffeate is higher than 5-hydroxyferulate (5-HFA) in contrast with angiosperm OMTs which will be discussed in the following chapters.

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p. 28)

The Lineweaver-Burk plots for the methylation of 5-HFA to SA in the presence or absence of caffeate gave typical competitive inhibition pattern, indicating that the SA-formation was greatly inhibited by caffeate (Figure 5). The Ki value, which should be equal to the Km value for 5-HFA, and other kinetic constants obtained by these plots were in good agreement with the values shown in Table 6. Further experiments indicated that SA-formation from 5-HFA (2.5×10^{-4} M) is completely inhibited by 2.3×10^{-5} M of caffeate added (Figure 6).

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DISCUSSION

The pine (Pinus thunbergii) OMT showed very poor SA-activity, which is good contrast to the angiosperm OMTs as summarized in chapter V. No inhibitors or activators were observed in the pine OMT during the purification, and hence the low SA/FA ratio belongs to the OMT itself. This well explains very low amounts of syringyl nuclei in the pine lignin, and Km values indicate that guaiacyl units are able to form smoothly in the conifer.

The role of OMT has been discussed with respect to the regulation of phenolase, while phenylalanine and cinnamate were examined as the regulating factors of OMT.[17,72] S-Adenosyl-L-homocystein is a potent inhibitor of this enzyme in plants[73-76] and animals.[77] The effect of illumination on OMT activity has also been investigated.[78-80] The kinetic studies in this experiment show that the SA-activity was competitively inhibited by caffeate. This finding indicates no sinapate-formation in the presence of caffeate in vivo. Thus, sinapate-formation is practically impossible in this plant because of the Km value for 5-HFA and the "feed-fore" inhibition. This clearly explains the phylogenic difference between gymnosperm and angiosperm lignins, the details of which will be discussed in chapter V. Recently, the enzymes responsible for the hydroxylation, activation and reduction of p-hydroxycinnamates

are found to be also important in determining the phylogenic difference of lignins.[51,61,69]

Concerning the OMT-polymorphism, it is necessary to ascertain whether or not the minor fraction in the crude enzyme preparation is a naturally occuring enzyme. In this point, it is interesting to consider the OMT activity in Pinus strobus and Pinus taeda which gave relatively high SA/FA ratios (chapter V). Thuja OMT was also reported to show higher SA-activity among gymnosperm OMTs, although the enzyme was concluded to be related to lignan biosynthesis.[68] The SA/FA ratio gradually decreased during the purification and the minor fraction of the enzyme was found in this experiment. However, conformational changes of the binding site, being known in a number of other enzymes,[81] might also explain the elimination of the SA-activity during the purification. Such possibility will be discussed in chapter IV.

In anion exchange chromatography, the gymnosperm OMT was eluted by lower KCl concentrations than bamboo OMT's(chapter IV). Major fraction of the pine OMT appears to be more positively charged comparing with aspen OMT (chapter II), bamboo OMT (chapter IV) and the minor fraction of the pine OMT on the polyacrylamide gel. Such positive charge of the major fraction might reflect some OMT-conformation which restricts the substrate specificity, i.e. preferential FA-formation with small SA-formation.

In conclusion, the methylation catalyzed by the pine OMT is an important step leading to the lignin which carrries almost entirely guaiacyl nuclei.

EXPERIMENTAL

Plant Material Pine (Pinus thunbergii) seedlings were grown at 25° C on wet vermiculite in the light at 12 hr intervals for 16 days. The primary xylem in the seedlings showed phloroglucinol-HCl positive color reaction.

Purification Procedure All purification procedures were performed at 4° C. Pine seedlings (545 g) were homogenized in the presence of polyclar AT (50 g) with equal weight of 0.1 M K-phosphate buffer (pH 7.5), containing 5 mM of each 2-mercaptoethanol, cystein, NaN_3 and iso-ascorbate. The homogenate was filtered through cheese-cloth and centrifuged at 17000 g for 30 minutes. The supernatant (920 ml) was adjusted to 60% saturation with solid ammonium sulfate in the presence of 5 mM of EDTA, and centrifuged at 7000 g for 30 minutes. The precipitate was dissolved in 0.02 M phosphate buffer (pH 7.4, 25 ml) and was passed through a Sephadex G25 column (42 x 3 cm). The desalted enzyme solution (108 ml) thus obtained was applied to a DEAE-cellulose column (3 x 7 cm) and eluted by 0.02 M K-phosphate buffer with step-wise increases in ionic strength using KCl. The proteins eluted by 0.1 and 0.15 M KCl (344 ml) were precipitated by the addition of solid ammonium sulfate with 5 mM of EDTA and caffeate (500 mg: anticipated to be a stabilizer of bamboo OMT, chapter IV). The precipitate was dissolved in the buffer (3.5 ml)

and the solution was then applied to a Sephadex G100 column (2.7 x 100 cpm; upward-flow rate 8.2 ml/hr; void volume 166 ml). The eluate (total volume 42 ml) was finally applied to a DEAE-cellulose column (3 x 1.3 m) with a linear gradient of from 0 to 0.2 M of KCl-buffer solution.

OMT Assay The reaction mixture (total volume 1 ml) for assay contained 0.1 ml of enzyme solution, 0.1 M of cystein, 2-mercaptoethanol and iso-ascorbate, 0.01 M $MgCl_2$, 0.2 ml of caffeate and 5-hydroxyferualte (0.5 µmole), 0.5 M K-phosphate buffer (pH 7.5). This reaction mixture without S-adenosyl-L-methionine-$^{14}CH_3$ (SAM; 0.25 µmole, 0.025 µCi) was preincubated for 5 minutes, the SAM was then added and the whole incubated for 0.5 -1.0 hour at 30° C. Extraction and the mesurement of the radioactivity were carried out as described in page 78. One unit (10^4 cpm) was equivalent to 53 nmole of product formed. Protein contents were determined by the mothod of Lowry et al.[82]

Disc Electrophoresis Disc electrophoresis was performed in a polyacrylamide gel according to Davis.[83] After electrophoresis the gel was cut lengthwise, and then into 5 mm thick discs. Each disc was crushed in the above reaction mixture, and FA- and SA-activities assayed as described above. The FA- and SA-activities of the discs at the purification step 2 was assayed for 3 hours at 30° C, using the same reaction mixture described above except the concentration of the

substrates was changed as follows, SAM (0.25 μCi), and caffeate (5×10^{-4} M) for FA-activity; SAM (0.1 μCi), and 5-hydroxy-ferulate (2.5×10^{-3} M) for SA-activity. Furthermore, the enzyme at step 5 was incubated for 2 hours with SAM (0.1 μCi), and substrates (5×10^{-4}M). The SA/FA ratios were calculated by subtracting 300 cpm in blank assay (minus substrate), except the case of SA-activity of the disc at the step 2, which was calculated by the following equation: SA-activity = (found activity - 300 x 2.5)/2.5 x 1.9, where 2.5 is the coefficient obtained from the specific activity of SAM, and 1.9 is the coefficient of the substrate concentration calculated from the Lineweaver-Burk plots, respectively.

Molecular Weight of the OMT The molecular weight was estimated by determining the elution volume on a calibrated Sephadex G100 column (1.4 x 88 cm; flow rate 8.5 ml/hour, K-phosphate buffer 0.05 M, pH 7.5), using the enzyme preparation at step 2.

Kinetic Studies of the OMT Competitive inhibition patterns were obtained from the reaction rate, when the concentration of 5-hydroxyferulate was changed from 8×10^{-4} to 5×10^{-3} M in the presence or absence of 5×10^{-5} and 10^{-4} M of caffeate. The enzyme assay was performed for 30 minutes, minimizing influence of the enzyme denaturation and product inhibition during the assay. When caffeate and 5-hydroxyferulate were incubated at the same time, the acids formed were separated by PPC developed in toluene-HOAc-H_2O (4:1:5, upper layer, decending method).

TABLE 3 PURIFICATION OF THE OMT IN THE PINE SEEDLINGS

PURIFICATION PROCEDURE	TOTAL PROTEIN (MG)	SPECIFIC ACTIVITY (UNITS/MG) FA	SA	RECOVERY (%) FA	PURIFICATION FA	SA/FA RATIO
HOMOGENATE	5 330	0.18	0.012	100	1.0	0.065
0-60% $(NH_4)_2SO_4$	1 079	0.62	0.041	70	3.5	0.066
DEAE-CELLULOSE	321	1.82	0.093	61	10	0.051
SEPHADEX G100	23	14.6	0.609	36	82	0.042
DEAE-CELLULOSE	13	15.9	0.588	22	89	0.037

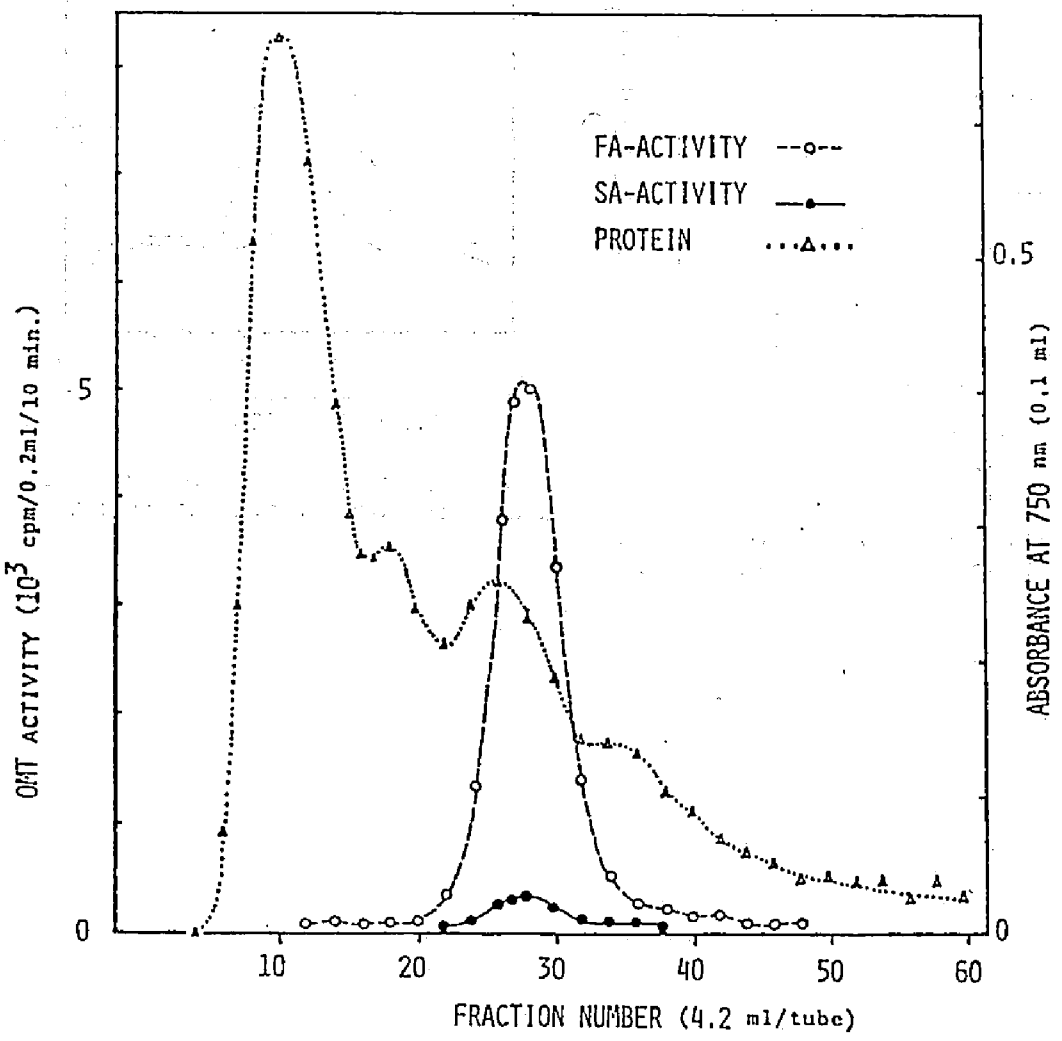

FIGURE 1 GEL FILTRATION PATTERN OF THE PINE OMT ON SEPHADEX G100 AT THE PURIFICATION STEP 4

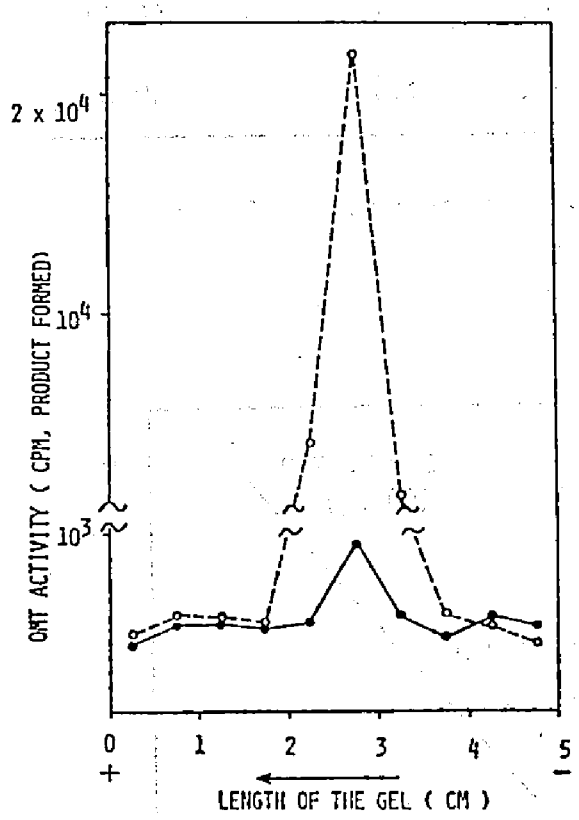

FIGURE 2 ELECTROPHORETIC PATTERNS OF THE PINE OMT ON POLYACRYLAMIDE GEL AT THE PURIFICATION STEP 5

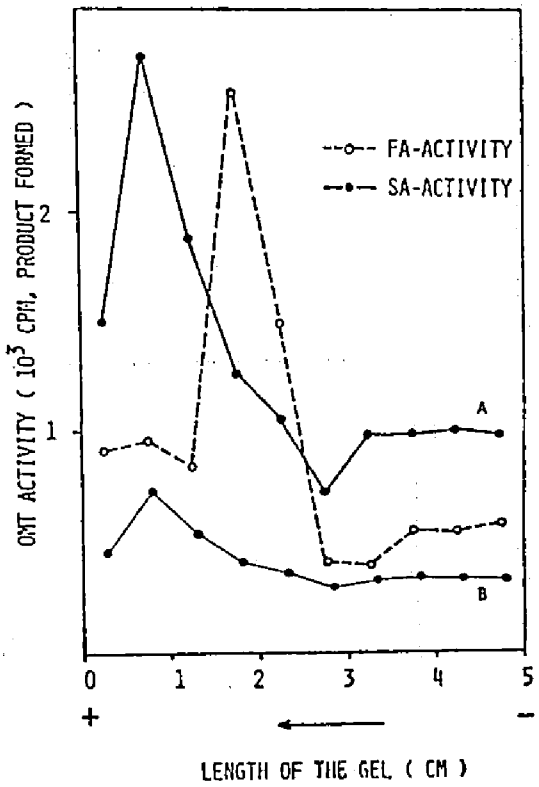

FIGURE 3 ELECTROPHORETIC PATTERNS OF THE PINE OMT ON POLYACRYLAMIDE GEL AT THE PURIFICATION STEP 2

A: FOUND ACTIVITY, B: CALCULATED ACTIVITY

SEE EXPERIMENTAL OF THIS CHAPTER.

TABLE 4 SA/FA RATIO OF THE MAJOR AND MINOR FRACTIONS OF THE PINE OMT AFTER DISC ELECTROPHORESIS ON POLYACRYLAMIDE GEL

OMT FRACTION	$(NH_4)_2SO_4$ PRECIPITATE (STEP 2) RELATIVE ACTIVITY FA	SA	RATIO SA/FA	FINAL STEP RATIO SA/FA
MAJOR	1	0.05	0.05	0.03
MINOR	0.30	0.18	0.6	—*

* Minor fraction was not observed.

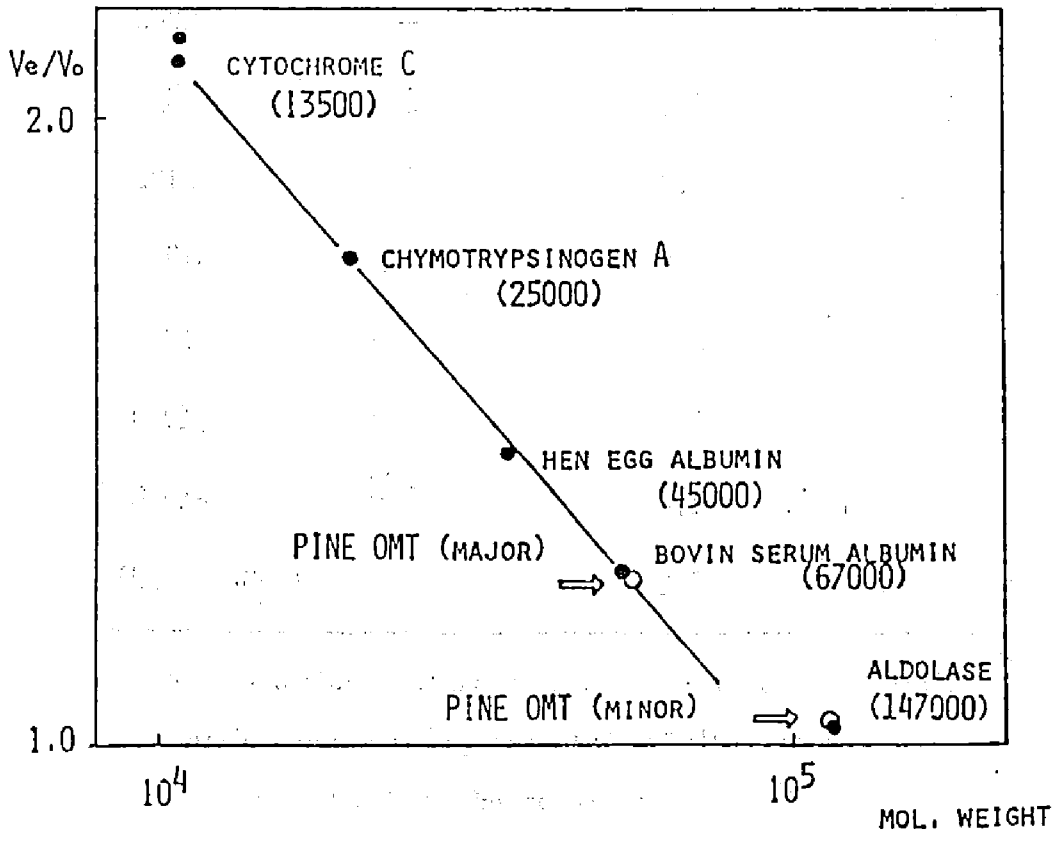

FIGURE 4 MOLECULAR WEIGHT OF THE PINE OMT DETERMINED BY GEL FILTRATION CHROMATOGRAPHY ON A CALIBRATED SEPHADEX G100 COLUMN

TABLE 5 EFFECT OF METAL IONS ON THE PURIFIED PINE OMT

ADDITION TO NONE*	CONCN. (mM)	METHYLATION RATE OF CA (10^3cpm/0.1 ml/hr)	RELATIVE RATE (%)
NONE*	—	7.8	80.1
NaF	0.5	9.8	99.8
EDTA	0.5	9.6	97.9
$MgCl_2$	1.0	9.8	100.0
$MgCl_2$	0.5	9.3	95.0
$CaCl_2$	0.5	9.5	96.9
$MnCl_2$	0.5	9.3	94.4
(LESS NaN_3**)	—	9.9	100.1

* The reaction mixture of NONE consists of the component which removed $MgCl_2$ from the complete system.

** removed NaN_3 from the complete system

TABLE 6 KINETIC CONSTANTS OF THE PINE OMT

SUBSTRATE	K_m (μM)	RELATIVE VALUE OF $1 / K_m$	V_{max} (cpm/ng/hr)	RELATIVE VALUE OF V_{max}
CAFFEIC ACID	51.1	5.4	66.1	24.8
5-HYDOXYFERULIC ACID	277	1.0	2.7	1.0
S-ADENOSYL-L-METHIONINE	40.6	6.8	69.9	26.2

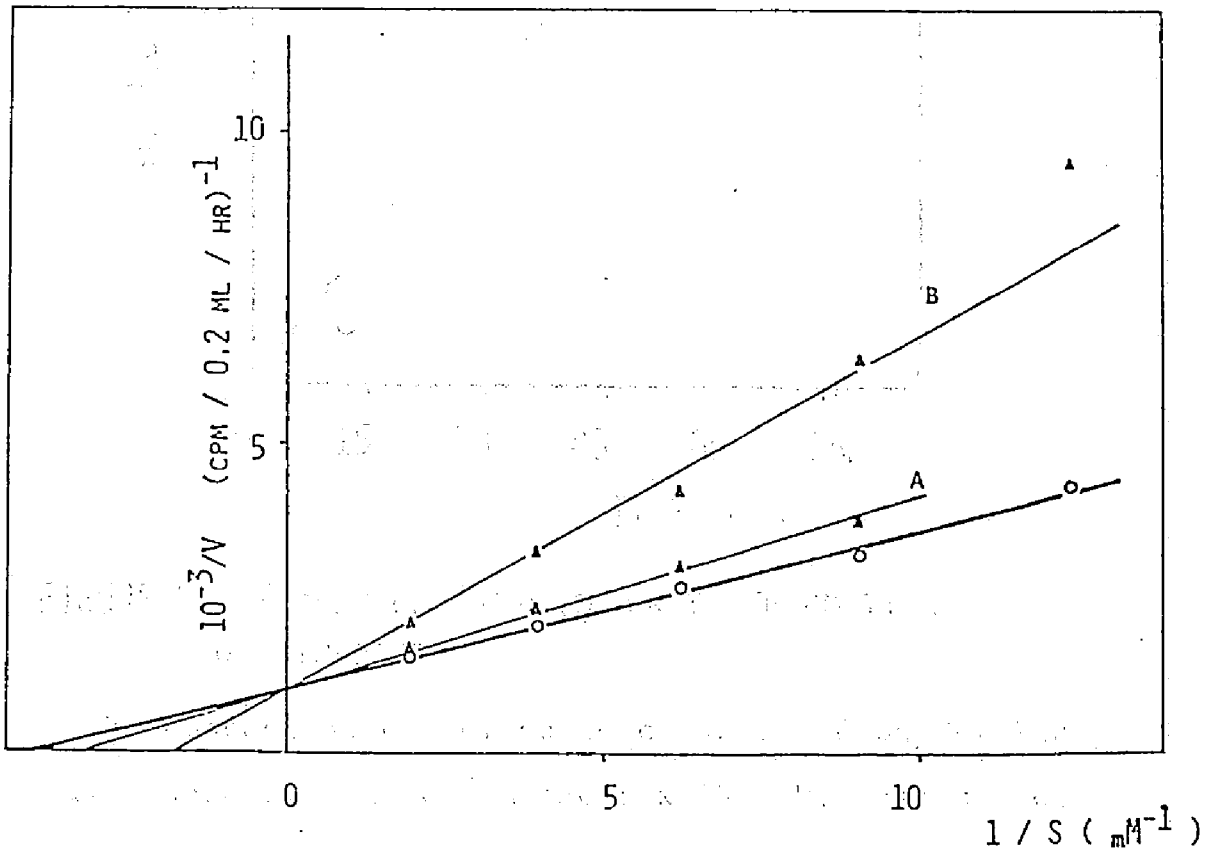

FIGURE 5 RECIPROCAL PLOT OF VELOCITY AGAINST 5-HYDROXYFERULATE IN THE PRESENCE AND ABSENCE OF CAFFEATE (A:10^{-5}M, B:5×10^{-5}M)

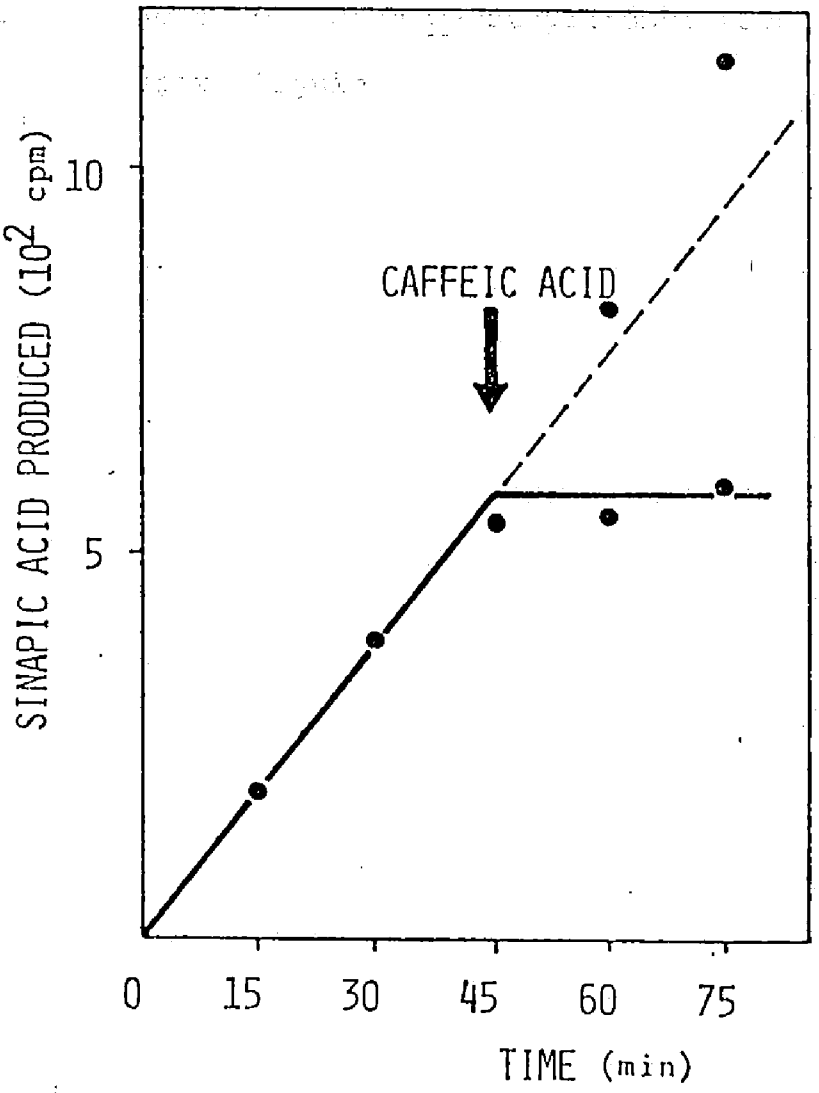

FIGURE 6 EFFECT OF CAFFEATE ON THE FORMATION OF SINAPATE

The concentrations of caffeic and 5-hydroxyferulic acids were 2.77×10^{-5} M and 5×10^{-4} M, respectively.

Chapter II Roles of Aspen O-Methyltransferase in Angiosperm Lignin.

INTRODUCTION

Shimada et al.[61] pointed out that plant OMTs are classified into two groups: one almost entirely catalyzes only guaiacyl-unit formation, and the other catalyzes both guaiacyl- and syringyl-unit formations. This finding well explains the reason why gymnosperm lignins are composed of only guaiacyl unit while angiosperm lignins are composed of both guaiacyl and syringyl units.

Several OMTs were characterized and their functions were discussed in relation to the biosynthesis of lignin,[61,67,75,84-88] flavonoids,[71,73,76,89] furanocoumarin[90] and o-, p-methylation.[89,91,92,93] The source used for the extraction and purification of OMTs were bud,[89] leaves,[74] roots,[87] young shoots,[84] seedlings,[67] and callus.[71,73,75,76,85,86,90,91] Various methylated phenolics in addition to lignin precursors often occur in the same tissues and multiple forms of the OMT were, in fact, reported to occur in plant tissues.[86,89-92] To characterize the OMT preferentially responsible for lignin biosynthesis, it is desirable to use the tissue which exclusively produces lignified cells, where the OMT is considered to be more directly involved in lignin biosynthesis. The trunks of big trees are suitable for this purpose. The isolation of cell-free enzymes from tree trunks has, however, scarcely been reported[94] because of the difficulty of the large scale extraction.

This chapter forcuses on the properties of OMTs from differentiating xylem tissues of ten-year old aspen trunks. The role of the angiosperm-type OMT is discussed in relation to the formation of guaiacyl and syringyl lignin precursors in dicotyledonous plants.

RESULTS

Evaluation of Aspen Trunks as an Enzyme Source

O-Methyltransferase (OMT) was extracted from the differentiating xylem tissue of aspen (Populus euramericana) trunks as described in the experimental. Rf values of the methylated products, i.e. ferulate and sinapate, were identical to those of the authentic compounds on paper and thin layer chromatograms using the solvent systems described in the experimental. It was found under microscopy that lysosome-like vesicles and vacuoles develop in differentiating xylem tissues of an aspen trunk (data not shown). The crude juice obtained from the phloem and young shoots of the aspen was rapidly turned brown even in the addition of polyclar AT. This might be ascribed to a high level of polyphenol oxidase activity in the crude extract of the phloem and shoots. The specific activity of the OMT extracted in the crude juice was reasonably high (10.6 pKat/mg protein for 5-hydroxyferulate) comparing with the activity reported on other plant materials for purification. The specific activities for caffeate of the starting OMTs usually lie between one or two pKat and several tens pKat per mg protein.[67,75,84,91] Although it was possible to improve the recovery of the enzyme by means of grinding the differentiating xylem with sea sand in a cold mortar, an alternative procedure using a Wiley mill for frozen wood

meal with liquid nitrogen was employed to extract the enzyme in a large scale. It was found that the differentiating xylem of aspen trunk was a good enzyme source for the large scale extraction.

Partial Purification of Aspen OMT

The apparent OMT polymorphism was observed when the crude OMT, fractionated by ammonium sulfate, was subjected to DEAE-cellulose chromatograph. Figure 7 shows elution profiles of the DEAE-cellulose column chromatograms, where two peaks were observed. The OMT eluted first from the column was observed to remain slightly in subsequent purification steps. It was found that the second peak has a tendency to increase (peak I) and the other relatively to decrease during the purification. The enzyme was considerably unstable during time consuming purification. It was unsuccessful to increase purification fold in spite of repeating experiments of the purification. Addition of cystein or EDTA was no effect on the stabilization of the enzyme, and a thorough purification was not achieved because of the instability of the enzyme.

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Table 7 summarizes one of the experiments in the purification of the aspen OMT. FA- and SA-activities show the formation of ferulate from caffeate and of sinpate from 5-hydroxyferulate, respectively. It was found that both activities in each peak of DEAE-cellulose chromatography were not resolved. The both

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activities were also unseparable by polyacrylamide gel electrophoresis at the final preparation (Figure 8), in which OMT was located to the peak I. The ratio of SA- to FA-activities, which is an indicator for the ability of sinapate formation to ferulate one by the enzyme,[61] remained constant (ca. 3) during the purification. It was found that the two peaks on the DEAE-cellulose chromatogram showed almost same SA/FA ratio (peak I 3.2; peak II 3.0): SA activities were not resolved from FA-activities in the both peaks.

Some Properties of Aspen OMT

The molecular weight of the peak I and II were almost same, and were estimated to be 72000 and 75000, respectively, by gel filtration chromatography. Optimal pH of the enzymes for FA- and SA-activities at step 2 was ca. 8.0, although the preparation was a mixture of peak I and II. Optimal pH for the activities at the final preparation was not measured, because the amount of the enzyme preparation was insufficient for other characterization and its instability.

Table 8 shows the effect of metal ions on the FA- and SA-activities of the final step preparation. Addition of Mg^{2+} (1 and 10 mM) was no effect on the enzyme activities. Other two valent metal ions (1 mM, respectively) were also no requisite on the full methylation rate. Addition of EDTA, NaF and NaN_3 (1,0.1,10 mM, respectively) did not inhibit the

enzyme activities, but moderately activated. The finding that the aspen OMT was not inhibited by EDTA and NaF, supports no requirment for Mg^{2+} ion in the enzyme activities. Heavy metal ions (1 mM, respectively) inhibited the methylation rates and the ions caused more than 90% inhibition when cystein in the reaction mixture was omitted (data not shown).

Table 9 shows that SH group is essential for the optimal catalytic activities. Thiourea and monoiodoacetate (0.1 mM, respectively) inhibited the activities, while cystein and mercaptoethanol were effective for the optimal methylation rates.

It was also found that the ratio of SA/FA activities were almost kept constant in these experiments.

Substrate Specificity of Aspen OMT

Table 10 shows the kinetic data of the OMT of the final step preparation. Km values for protocatechualdehyde, 5-hydroxy-vanillin, caffeate and 5-hydroxyferulate were found to be of the order of 10^{-4} M. Vmax/Km for 5-hydroxyferualte was 5.4 times greater than that for caffeate. Protocatechualdehyde was a better substrate than caffeate. The kinetic data indicate that the formation of syringyl unit is preferential than that of guaiacyl unit. The other o-diphenolic substrates examined were quercetin, cyanin, catechin, protocatechuic acid and pyrocatechol. The methylation rate for these substrates

was less than 2% of that for caffeate. Almost no methylation were observed in the case of other phenols, i.e. apigenin, kaempferol, 3,5-, 2,4-, 2,6-, 2,5-dihydroxybenzoates, saligenin, salicin, salicylaldehyde, arbutin, o-, p-coumarate, ferulate, iso-ferulate and sinapate.

DISCUSSION

Multiple forms of plant OMTs were reported by several authors.[86,89-92] It was shown that Populus glandular tissue contained a few OMTs.[89] We also observed polymorphism of the OMT in the differentiating xylem of an aspen trunk. It is interesting to consider this point in relation to the finding that the differentiating xylem cell contains many lysosome-like vesicles and developing vacuoles. It seems likely that the proteases occur in such organelles of a differentiating stage, in order to redistribute nitrogen from lignified xylem cells to cambium in tree trunks. Such phenomenon was reported during grain growth in wheat.[95] Therefore, proteases of the organelles might cause lability and modification of aspen OMT when the tissue was ground and subjected to a rather time consuming enzyme purification process (Figure 7). Such assumption is in accord with the result that the molecular weight of the peak II was slightly smaller than that of the peak I. It must be kept in mind that multiple forms of the reported OMTs might be produced during the extraction and purification. Such example of the OMT was reported on protein patterns obtained by gel electrophoresis at each purification step in tabacoo suspension cells.[91] It is also assumed that the polymorphism observed is not an artifact but reflects some physiological role *in vivo*. At present, there is no direct evidence

to show multiplicity of the aspen OMT, and further research is needed to clarified these problems.

It is difficult for plant OMTs to find the general correlations between their biosynthetic role and properties. The properties of an OMT in different plants were not always consistent even if the enzyme plays the same biosynthetic role. Purified preparation of an OMT does not always show the same properties as the crude one in an enzyme source. These make it difficult to describe the general properties of an OMT accurately, but plant OMTs might be roughly characterized as follows. The optimal pH of caffeate-specific OMTs was ranged from pH ca. 6.5 to 8.0.[65,67,75,84,87] Lignin-specific OMTs generally do not require Mg^{2+} ion[67,75] except the case of bamboo.[96] This is in good contrast to that of flavonoid-specific OMTs in parsley and soybean cell suspension culture,[71,76] which absolutely required Mg^{2+} ion. Sulfhydryl group inhibitors generally inhibit plant OMTs in various extents[67,73,90,91,96] except flavonoid-specific parsley OMT which was not affected by the inhibitors.[71] Effect of EDTA on the OMTs is not always constant: Lignin-specific OMTs showed no effect[75,90] but another lignin-specific one was moderately activated by the chemical.[67] Flavonoid-specific OMT was not affected by EDTA.[90] Molecular weight of plant OMTs reported lie between 40000 and 110000.[67,71,73,90,91] It was found that the aspen OMT characterized in this chapter fills the general properties

of lignin-specific OMT as described above.

It is noteworthy that the catechols with C_1-side chains of aldehyde and alcohol were as good substrates as caffeate. The finding indicates that the enzyme might be involved in the methylation of C_6-C_1 diphenols. However, almost no methylation of protocatechic acid was observed although vanillic and syringic acids are widely distributed in this genus. Such substrate specificity of the OMT seems to be affected by the functional groups of the C_1-side chains. This findings might be expanded to diphenolic C_6-C_3 lignin precursors. It is demonstrated that the cinnamate derivatives are reduced to the corresponding alcohols via CoA-esters and aldehydes during lignification.[20] The C_6-C_3 o-diphenolic substrates carring these side chains might be methylated by a lignin-specific OMT, although caffeoyl-CoA was not methylated by the OMT.[75] The cell free system involved in the reduction of sinapate to sinapyl alcohol was demonstrated, but the universal process of syringyl lignin formation is still obscure.[51] Further survey of the substrate specificity for the OMT is desired to clarify the lignin biosynthetic pathway, especially the formation of syringyl lignins.

The results in the present investigation is concluded as follows. First, the characterized aspen OMT is related to lignin biosynthesis, because of a defined phenolic metabolism in the xylem tissues and of substrate specificities of the

enzyme. The cambium of aspen trunks differentiates secondary xylem, where lignin polymers are present with small amounts of lignin precursors. Since phenolic metabolism in the differentiating xylem tissues considerd to be exclusively directed to lignin formation, the OMT in these xylem cells could be involved in the methylation of lignin precursors. The substrate specificities of plant OMTs have been discussed from the standpoint of lignification, flavonoid biosynthesis and o-, p-O-methylation. It was found that the aspen OMT can not methylate flavonoids nor p-position in the phenolic substrates surveyed. These substrate specificities strongly suggest that the OMT is related to lignin biosynthesis in the xylem of aspen trunks. Second, the aspen OMT probably operates as a "fine ajustment" enzyme in guaiacyl and syringyl lignin formation. This is led from the findings that the same OMT catalyzes the formation of both guaiacyl and syringyl nuclei with preferential formation of the latter nuclei. If caffeate and 5-hydroxylferulate are formed in a same site of a xylem cell, the latter substrate will be preferentially methylated by the OMT until the substrate will reduce to the level which do not interfere the former methylation. The aspen OMT may control guaiacyl and syringyl lignin formation in this way, i.e. fine adjustment which is capable of very minute alteration of the both units. The fine and coarse adjustments seem to be operated in the increasing ratio of

syringyl to guaiacyl nuclei during the development of the xylem in dicotyledonous plants.[88,97] The latter adjustment might be also operated by the enzymes participating in the reduction of p-hydroxycinnamates to corresponding alcohols. Last, the substrate specificities of aspen OMT explain a part of the reason why aspen lignin contains much syringyl nuclei comparing with gymnosperm one. This property is generally applicable to the angiosperm OMTs which related to lignin biosynthesis. Interesting examples were reported in swede root,[87] Erythrina[98] and mistletoe[99] (chapter III). The swede root OMT was found to show low SA-activity. These points will be discussed in chapter V.

The physiological roles of lignin-specific OMTs are significant both in the differentiation and in the phylogenic difference of the SA/FA ratio, i.e. the formation ability of syringyl and guaiacyl nuclei.

EXPERIMENTAL

Materials S-Adenosyl-L-methionine-$^{14}CH_3$ (SAM: 45.9 mCi/m mol) was purchased from New England Nuclear, and diluted with unlabelled SAM. The diluted SAM was, then, purified on paper chromatogram using 5% HOAc in 80% EtOH at 4-6° C (specific activity of the SAM: 0.3 mCi/m mol). A protein kit for the calibration of molecular weights, and cold SAM were obtained from Boehringer Mannheim GmbH. Hydroxycinnamates were synthesized from corresponding benzaldehyde and malonic acid.[100] 5-Hydroxyvanillin was prepared in the conventional way.[101] Flavonoids used for the substrate specificity were obtained from Tokyo Kasei Co.,Ltd. 5-Hydroxyvanillyl alcohol was prepared by Mr. Kutsuki of the Research Section of Lignin Chemistry, Wood Research Institute, Kyoto University.

Plant Source for the OMT Extraction Two ten-year-old aspen (Populus euramericana) trees were cut down in June and the trunks free of the bark were used for the enzyme source. The differentiating secondary xylem was scraped by a scraper and the chips were immediately frozen in liquid nitrogen. The wood frozen was milled with continuous addition of liquid nitrogen in a Wiley mill, and stored at -20° C until for use.

Chromatographic Methods for the Identification of the Methylated Products The methylated products formed were co-chromatographed on paper chromatogram with the solvent system: (1)

toluene-HOAc-H_2O (4:1:5, v/v, organic layer), (2) $CHCl_3$-HOAc-H_2O (2:1:1, v/v, organic layer) and thin layer chromatogram of silica gel with the solvent system: MeOH-isopropyl ether (1:10, v/v).

OMT Assay Standard reaction mixture contains phenolic substrates (0.2 µmol), $MgCl_2$ (0.2 µmol), Tris buffer (pH8.0, 10 µmol), enzyme solution (up to ca. 50 mg of fresh weight) and SAM (0.03 µCi/0.1 µmol). The SAM was added as a starter after preincubation for 5 minutes (total volume 200 µl). The mixture was incubated for 10, 20 and 30 minutes at 30° C with or without phenolic substrates. The reaction was terminated by the addition of 2 N HCl (20 µl) and extracted with $CHCl_3$ or EtOAc (the latter, for the extraction of flavonoids). The methylation rate was determined by the time course of the radioactivity in the extract. Two ml of scintillator, which contains 2,5-diphenyloxazole (4 g) and 1,4-Bis[2-(4-methyl-5-phenyloxazole-2-yl]benzene (0.1 g) in toluene (1.0 l), was used for the scintillation spectrometry.

OMT Extraction and Purificaiton Step 1: The wood meal (615 g) was extracted with 1.7 l of 0.2 M K-phosphate buffer (pH 7.7) containing 5 mM of 2-mercaptoethanol and NaN_3 by a ultratrax homogenizer. All procedures of the extraction and purification were performed in a cold room at 4-6° C. The homogenate was squeezed with a four layered guaze and the filtrate was centrifuged at 9000 rpm for 20 minutes.

Step 2: The supernatant solution (1.2 l) was fractionated with solid ammonium sulfate while the pH was maintained at 7.7 and the protein precipitated between 20 and 55% saturation was collected by centrifugation. The precipitate was re-suspended in a minimum volum of the K-phosphate buffer and dialyzed for 8 hours against the same buffer (total volume 77 ml).

Step 3: The desalted solution was applied on a DEAE-cellulose column (2.2 x 11 cm) previously equilibrated with the K-phosphate buffer. Proteins were eluted with a linear salt gradient between 0 and 0.5 M KCl in the K-phosphate buffer after washing with 50 ml of the K-phosphate buffer. The peak I and II were eluted at ca. 0.1 and 0.2 M KCl, respectively. Fractions of 60 drops were collected (peak I and II: 49 and 84 ml, respectively).

Step 4: The two peaks were concentrated by the addition of ammonium sulfate (90% saturation) and the precipitated proteins were dissolved in a minimum amount of the K-phosphate buffer. The each solution was applied on a Sephadex G200 column (1.55 x 89 cm) which was equilibrated with the K-phosphate buffer. The molecular weights of the enzyme were calibrated using this column. The flow rate was at 10 ml/hr and the 30 drops of each fraction was collected. The volume of the peak I and II collected were 34 and 33 ml, respectively.

Step 5: The combined fractions (peak I and II) were

absorbed on a hydroxyapatite column (1.55 x 7.2 cm) which was bufferized with 0.002 M K-phosphate buffer (pH 6.7) containing 5 mM of 2-mercaptoethanol. The proteins were eluted with a linear gradient between 0.002 and 0.2 M K-phosphate buffer containing 5 mM of 2-mercaptoethanol. Fractions of 40 drops were collected. OMT activity was not resolved by this procedure but a shoulder of the elution profile was observed. proteins were determined by the Folin method of Lowry et al.[82]

Disc Electrophoresis Disc electrophoresis was carried out by the method of Davis.[83] The gel, after the electrophoresis, was cut into 2 mm thick. Each half of the slices was directly assayed for FA- and SA-activities.

TABLE 7 PARTIAL PURIFICATION OF ASPEN OMT

PURIFICATION STEP*	PROTEIN (MG)	TOTAL ACTIVITY (NKAT)		SPECIFIC ACTIVITY (PKAT/MG)		RATIO	RECOVERY (%)	FOLD
		SA	FA	SA	FA	SA/FA		
STEP 1	461	4.89	1.56	10.6	3.38	3.14	100	1.00
STEP 2	159	3.42	1.06	21.5	6.68	3.22	69.9	2.03
STEP 3	31.3	2.57	0.786	82.0	25.1	3.27	52.5	7.92
PEAK I	12.9	2.26	0.685	175	53.0	3.30		
PEAK II	18.4	0.310	0.103	16.8	5.56	3.02		
STEP 4	12.4	1.67	0.522	135	42.2	3.20	34.1	12.7
PEAK I	5.80	1.30	0.400	224	68.9	3.25		
PEAK II	6.60	0.371	0.122	56.1	18.5	3.04		
STEP 5	3.71	0.677	0.214	183	57.9	3.16	13.9	17.3

* STEP 1: CRUDE EXTRACT, STEP 2: AMMONIUM SULFATE FRACTIONATION, STEP 3: DEAE-CELLULOSE CHROMATOGRAPHY, STEP 4: SEPHADEX G200 CHROMATOGRAPHY, STEP 5: HYDROXYAPATITE CHROMATOGRAPHY (SEE ALSO EXPERIMENTAL IN THIS CHAPTER)

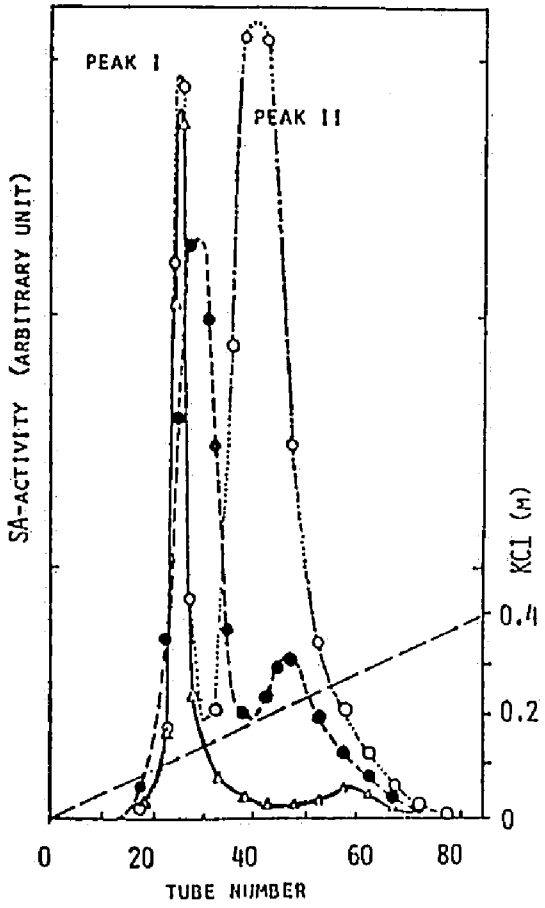

FIGURE 7 ELUTION PROFILES OF ASPEN OMTS FROM DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

—o— A CRUDE JUICE CORRESPONDING TO STEP 1.
—●— STEP 3 IN THE TABLE 7. —△— RE-CHROMATOGRAM AFTER STEP 3 (KEPT IN A REFRIGERATOR MORE THAN 2 WEEKS).

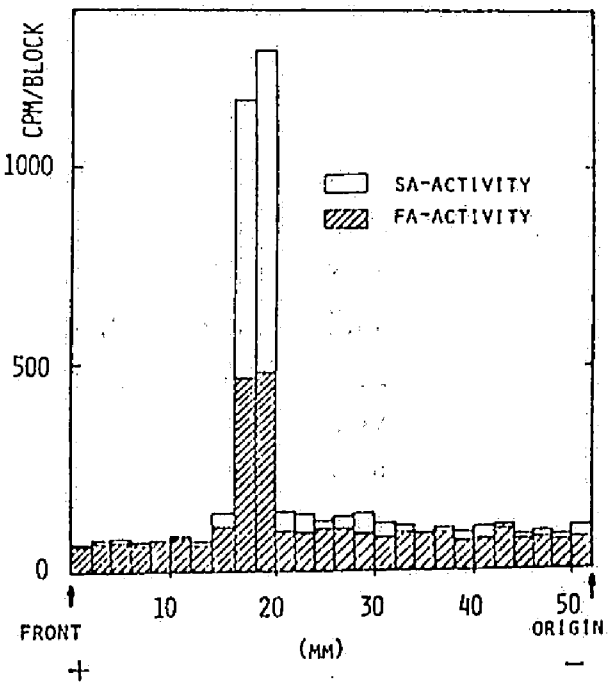

FIGURE 8 DISC ELECTROPHORETIC PATTERN OF ASPEN OMT AT THE PURIFICATION STEP 5

FA- AND SA-ACTIVITIES IN AN OMT WERE NOT SEPARATED BY THE GEL ELECTROPHORESIS AT THE FINAL PURIFICATION STEP. THE CONTROL (REACTION MIXTURE MINUS SUBSTRATE) WAS NOT SUBTRACTED FROM THE BOTH ACTIVITIES ILLUSTRATED.

TABLE 8 EFFECT OF METAL IONS ON ASPEN OMT

SYSTEM*	RELATIVE		RATIO
	FA	SA	SA/FA
STANDARD	100	100	3.15
$MgCl_2$ (1 mM)	100.2	100.6	3.16
$CaCl_2$ (1 mM)	101.4	96.8	3.01
$MnCl_2$ (1 mM)	88.8	83.0	2.95
$BaCl_2$ (1 mM)	104.8	102.1	3.07
$NiCl_2$ (1 mM)	83.4	77.2	2.91
$ZnCl_2$ (1 mM)	63.7	63.1	3.12
$CoCl_2$ (1 mM)	72.8	67.0	2.90
$HgCl_2$ (1 mM)	73.9	72.0	3.07
NaN_3 (10 mM)	113.2	113.4	3.16
NaF (0.1mM)	117.8	117.3	3.14
EDTA (1 mM)	114.5	107.8	2.97

* THE SYSTEM CONTAINS STANDARD REACTION MIXTURE (SEE ALSO EXPERIMENTAL IN THIS CHAPTER) EXCEPT THE ADDED CHEMICALS AS A SUBSTITUTE FOR Mg^{2+}. WHEN NaF WAS ADDED TO THE SYSTEM, K-PHOSPHATE BUFFER WAS USED INSTEAD OF TRIS-BUFFER.

TABLE 9 PARTICIPATION OF SH-GROUP IN THE ENZYMIC METHYLATION

SYSTEM*	SA (pKAT/MG)	FA (pKAT/MG)	RATIO SA/FA	RELATIVE SA
STANDARD	171	54.8	3.12	100
MINUS $MgCl_2$ MRSH CYSH	36.4	11.8	3.98	21.3
MINUS MRSH CYSH	37.4	11.9	3.14	21.9
MINUS $MgCl_2$ MRSH	172	55.3	3.11	100.6
MINUS $MgCl_2$ CYSH	146	47.9	3.05	85.4
PLUS PCMB (0.1 MM)	3.59	2.79	1.29	2.10
PLUS THIOUREA (0.1 MM)	32.1	11.2	2.87	18.8

* MRSH: 2-MERCAPTOETHANOL, CYSH: CYSTEIN, PCMB: P-MERCURIC BENZOATE

TABLE 10 KM, RELATIVE VMAX AND VMAX/KM VALUES OF ASPEN OMT

SUBSTRATE	KM (10^{-4}M)	RELATIVE VMAX	RELATIVE VMAX/KM
CAFFEATE	3.8	1.0	1.0
PROTOCATECHU-ALDEHYDE	2.6	2.6	3.8
5-HYDROXYVANILLYL ALCOHOL	3.4	3.7	4.1
5-HYDROXYVANILLIN	4.8	3.5	2.8
5-HYDROXYFERULATE	3.1	4.4	5.4

Chapter III Biosynthesis of Mistletoe Lignin and the Roles of the O-Methyltransferase.

INTRODUCTION

Mistletoe(Viscum album) is an evergreen dicotyledon which is usually parasitic on Celtis sinensis and occasionally on cherry trees in Japan. Several characteristic compounds such as physiologically active proteins,[102-104] viscotoxine,[105,106] acetylcholine,[107] verazine,[108] a cyclic peptide[109] and flavonoids[110-112] were isolated from mistletoes.

On the other hand, a few studies have been reported on the nutrition dependence of mistletoe on the hosts.[113-115] Mistletoe usually gets water from the host, and some species are known to be depend carbon sources as well on the host.[113-115] Freudenberg reported that European mistletoe grown on either gymnopserm or angiosperm trees gave the corresponding type lignins.[116] However, the lignins in European mistletoe lately demonstrated to be angiosperm type one independent on the host trees.[117-119] It has been further reported that flavonoids in a parasitic plant are quite different from those of the host plants.[110,112] These observations may indicate that the biosynthesis of mistletoe lignin is generally controlled by the parasitic plant itself and not by host trees.

However, the direct demonstration about such nutrition dependency of the lignin biosynthesis was still missing. Actually, mistletoe O-methyltransferase did not catalyze ferulate-formation, suggesting no lignin biosythesis in the

plant. The mistletoe grown on Pinus silvestris tree was found to be rather less abundant in syringyl lignin.[118,119] This chapter describes chemical properties of parasitic and host lignins, and elucidates if the mistletoe plant produces lignins independently from the host. The enzyme in cinnamate pathway, especially O-methyltransferase is also discussed.

RESULTS AND DISCUSSION

Characterization of Mistletoe Lignin

Analytical results of mistletoe (Viscum album) lignin was compared with the host (Celtis sinensis) ones, and the data obtained are summarized in Table 11.

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Lignin contents in the mistletoe and host woods were estimated to be 22.1 and 21.6% by Klason method, respectively. The values showed typical angiosperm ones in normal woods. Emperical formulae of the milled wood lignins (MWLs) showed rather high oxygen contents in comparison with Freudenberg's results.[116] Since the MWLs in this experiment were purified by the Björkman's standard method,[120] the lignins probably contained some contaminated materirals such as sugars which may cause high oxygen contents in the formulae.

Infrared spectra of the mistletoe and host MWLs gave almost identical absorption bands. The main bands observed were 1325, 1235, 1130 cm^{-1} (syringyl ring); 1275, 11451 1030 cm^{-1} (guaiacyl ring); 1595, 1505, 1425 cm^{-1} (aromatic skeletal bands) and the bands assigned to aliphatic and aromatic bonds.[121] Mistletoe MWL showed higher relative absorptivities than the host one at the band ratios 1665/1505, 1325/1505, 1230/1275, and 1130/1030 except the ratio 1735/1505 which shows the amount of unconjugated ester carbonyl groups.[122] The relative ratio (1600/1505) is known to be increased by the presence

of syringyl component, p-hydroxyphenyl esters, carboxylate ions in carbohydrates and condensed tannin impurities.[121] However, the higher value at 1600/1505 in mistletoe MWL seems to be mainly ascribed to the predominace of syringyl component considering the methoxyl contents and the ratios (1325/1505, 1230/1275, 1130/1030). Furthermore, the presence of conjugated ester groups, such as p-coumaryl esters observed in UV spectrum of bamboo lignin,[123] was neglibible in the MWLs. The UV spectra in the MWLs of the mistletoe and the host showed maxima at 277.5 nm (absorptivity 15.3 l/g-cm) and 278.0 nm (absorptivity 14.4 l/g-cm), respectively. No absorption was observed in the region around 350 nm, the absorption at which is due to the presence of the conjugated ester groups, and the spectra in this region showed a general absorption curve. These observations thus indicated that both mistletoe and host lignins are typical angiosperm type ones, although some variation was observed in the intensities of IR region.

The MWLs were degraded by acidolysis, and then the monomeric products were analyzed to confirm the given information in the spectral analyses. The yields of acidolysis oils were 58 and 61% of the mistletoe and host MWLs, respectively. The ratios of syringyl acetone, (vanilloyl methyl ketone and syringoyl methyl ketone) to guaiacyl acetone in the acidolysis of mistletoe and host lignins were 0.92 (0.38 and 0.18) and (0.27 and 0.11), respectively. These values showed that

both lignins are comparable to a typical angiosperm one: i.e. the corresponding values of beech wood were 0.71, 0.38 and 0.15. The following compounds were identified by GC-MASS analyses as TMS-derivatives of the acidolysis products of the MWLs and a beech wood (control); guaiacyl acetone, vanilloyl methyl ketone, syringyl acetone and syringoyl methyl ketone. A few other peaks which appeared faster than guaiacyl and syringyl derivatives were observed on gas chromatograms in all cases. These peaks were very small in their areas except rather large one, M^+ 220 which was identical with M^+ of trimethyl syrilated p-coumaryl aldehyde but showed a little different fragment ions from those of the aldehyde. p-Hydroxyphenyl derivatives may not be ascribed to these peaks, because they were observed not only in the acidolysis products of beech wood, but also mass spectra of the p-hydroxyphenyl monomers,[124] such as p-hydroxybenzaldehyde, p-hydroxyphenylacetone, 1-hydroxy-1-(4-hydroxyphenyl)-2-propanone and p-hydroxybenzoyl methyl ketone, were different from those of these peaks. The analytical results indicated that both mistletoe and host lignins are typical angiosperm type, although the mistletoe lignin gave some degradation products which were in small amount and could not be identified.

Recently, FT-^{13}C-NMR was introduced into the structural studies of lignins and showed to give many useful informations.[119, 125-129] Thus, the method was applied to the mistletoe lignin and

the MWLs, which gave typical angiosperm-type spectra in aromatic and aliphatic carbon regions. Ester carbonyl carbon (172.4 ppm from TMS)[126] which was not observed in the mistletoe lignin, was observed in the host MWL in accordance with the absorption ratio (1735/1505) in IR region. The absorption peaks of guaiacyl and syringyl ring carbons were observed in both mistletoe and host MWLs. The peaks at 154.3, 139.3, 135.9, 107.7 and 105.7 ppm from TMS internal standard observed in these MWLs may be assigned to the absorption of syringyl ring carbons on the position 3 and 5, 1 and 4, 4, 2 and 6, 2 and 6, respectively.[126,127] No distinct peaks appeared in the region of p-hydroxyphenyl ring carbons (160.9, 131.3 and 117.0 ppm from TMS, control:bamboo MWL) in these MWLs.

Thus, the mistletoe lignin as well as the host lignin were confirmed to be typical normal angiosperm type ones.

Mistletoe O-Methyltransferase and Lignin Biosynthesis

O-Methyltransferases (OMTs) involving in the biosynthesis of angiosperm lignin catalyze the formation of ferulate (FA) and sinapate (SA) from caffeate and 5-hydroxyferulate, respectively,[84] while the gymnosperm ones hardly catalyze the formation of SA.[67] The ratio of SA- to FA-activities (SA/FA) is very useful as an indicator for the ability of syringyl lignin formation.[61] As it will be discussed in chapter V, the phylogenic distribution of gymnosperm and angiosperm lignins

is partially ascribed to the ratio SA/FA of these OMTs.

The SA/FA ratio of the mistletoe homogenate was quite different from these generalization and showed remarkably high value as a result of negligible FA-formation. On the basis of the biosynthesis of lignin monomers in mistletoe, no FA-formation contradicts the results which showed the mistletoe lignin as a typical angiosperm-type irrespective of the host species.[118,119] Hence, the cause of the no detection of FA-formation should be considered as follows; i) the presence of a particular OMT which is unable to catalyze the FA-formation. ii) normal angiosperm-type OMT is present but FA-activity is inhibited by some causes. Each case above mentioned may be understood in the following way. In the case of i), 1.1. Mistletoe has no ability to synthesize lignin itself. 1.2. Mistletoe is able to synthesize lignin monomers, but the biosynthetic pathway is different from normal angiosperm one. In the case of ii), 2.1. Mistletoe has no ability to synthesize lignin itself because FA-activity is inhibited. 2.2. Normal angiosperm-type pathway in lignin biosynthesis is present in mistletoe, but the FA-activity is inhibited by some inhibitors which may be liberated by the breakage of the compartmentation in the cell during the OMT extraction.

Thus, the mistletoe OMT was purified to elucidate the

cause of the no FA-activity in relation to the ability of lignin biosynthesis. The SA-activity of the mistletoe OMT in crude homogenate showed optimal pH around 7.2, FA-activity at which was not detected. However, a weak FA-activity optim pH of which is 5.5 was observed in the lower pH. The FA-activity was detected even at pH 7.5 after ammonium sulfate precipitation followed by dialysis of the crude extract. The purification results of the mistletoe OMT are shown in Table 12. The FA-activity was remarkably activated by dialysis followed by the treatment of DEAE-cellulose, and the ratio SA/FA (2.6) became small as in common angiosperm one (the ratio of the host, 2.7).

le 12
p. 66)

Since the presence of specific substrate(s) inhibiting FA activity was suggested, the DEAE-cellulose after elution of the OMT was treated with 0.05 M K-phosphate buffer (pH 5.2) and then the eluate was adjusted to pH 7.0. This fraction inhibited not only the FA-activity of mistletoe enzyme but also that of gymnosperm (Pinus thunbergii) and angiosperm (Pueraria Thunbergiana) OMTs, although SA-activities in these plants were gradually inhibited by aging of the fraction. The aged fraction was able to inhibit the both activities even by treatment of heating (100° C, 5 minute) or by pronase treatment. No inhibition was observed in the inhibitor fraction after dialysis against 0.025 M K-phosphate buffer (pH 7.4) for 8 hours. It is noteworthy that β-glucosidase[130]

and glucan phospholyrase[131,132] activities were inhibited by the addition of crude mistletoe juice, and the latter inhibition was suggested to be caused by some phenols.[132] The inhibition of OMT may not be caused by an ortho-phenol which is a methyl acceptor of S-adenosyl-L-methionine and compete with caffeate or 5-hydroxyferualte, because no methylation was found in control assay (minus substrate) in these experiments. Although the characterization of the inhibitior(s) is incomplete, it seems that the mistletoe OMT is a normal angiosperm one and that the methylation of caffeate to ferulate is not blocked in vivo. Thus, the assumption 1.1. and 1.2. described above were denied.

Biosynthesis of Mistletoe Lignin

The ability of mistletoe to synthesize lignin was evaluated by the other enzyme reactions relating to lignin biosynthesis. Phenylalanine ammonia-lyase (PAL) was not detected in the intact mistletoe plant even in early July in which the high activity was expected. Figure 9 shows the development of enzyme activities during the incubation of the sliced mistletoe tissues under illumination. PAL activity, the product of which was identified to be trans-cinnamate by GC-MASS spectrometry, was gradually activated but the FA-activity of the OMT was not detected during the incubation, showing that the inhibitor(s) of OMT did not affect PAL activity. In addition, it was suggested

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p. 67)

that the OMT and PAL may be under different genetic control because of the different activation pattern similar to those of parsley cell suspension culture.[78]

Cinnamate-4-hydroxylase activity, which was very low in this plant, was detected and the product formed was also identified by mass spectrometry, and the p-coumarate was not detected in control assay (minus substrate).

Overall enzyme reations in lignin biosynthesis were examined by tracer experiment using phenylalanine-U-^{14}C. The plant fed was allowed to metabolize for 24 hours and then the plant tissues were oxidized after extraction with 80% hot ethanol and ethanol-benzene (1 : 2),successively. The results shown in Table 13 indicate that mistletoe is able to synthesize both guaiacyl and syringyl nuclei, and that the ratio of the oxidation products (syringaldehyde/vanillin) is in accord with that of the products of normal angiosperm lignin. The high yield of p-hydroxybenzaldehyde as an oxidation product was found in the mistletoe. However, it is suggested that the aldehyde may not be derived from the mistletoe lignin, because of its low specific activity and of no evidence for the presence of p-hydroxyphenyl nuclei in the lignin. The origin of p-hydroxybenzaldehyde in the oxidation products of the mistletoe remains obscure at the present time.

ble 13
(p. 67)

All the results obtained concluded that the mistletoe has, at least, enzyme systems from phenylalanine to lignin and is able to synthesize the angiosperm-type lignin.

EXPERIMENTAL

Plant Materials Lignified wood tissues of the mistletoe (Viscum album) and the host (Celtis sinensis), which were grown in Uji city, were used for preparation of milled wood lignin. Fresh shoots of the mistletoe were used for the enzyme assay, and tracer experiment after removal of the leaves, respectively.

Preparation of Milled Wood Lignins (MWLs) Extractive free-wood meal (40 mesh) was fully dried over P_2O_5 in vacuo, and milled in toluene for 100 hours using a vibration ball mill in a usual way.[120] The milled wood powder was extracted with dioxane/water (9:1), and the MWL extracted was dissolved in HOAc/H_2O (9:1), and then the soluble portion was dropped into water. The MWL thus obtained was further purified by dropping it into Et_2O after dissolution in dichloroethane/EtOH (2:1). The MWL was then collected by centrifugation and dried completely over P_2O_5 in vacuo.

Estimation of Methoxyl Groups of the Lignins A conventional volumetric method was employed.[133]

Spectral Analyses of the Lignins UV and IR spectra were measured in dioxane/H_2O (9:1) and in KBr wafer, respectively. FT ^{13}C-NMR spectra were measured in $(CD_3)_2CO/D_2O$ (9:1) by scanning 5.3×10^4 times (100 MHz).

Acidolysis of MWLs The MWL (10 mg) was dissolved in 1 ml of

2 N HCl/dioxane (9:1) and the solution was sealed in a glass tube after flushed with N_2 gas. The reaction mixture was kept at 120° C for 20 hours, and then the mixture was dropped into water (15 ml) and extracted with Et_2O. The acidolysis oil thus obtained was trimethylsyrilated in usual way[134] and analyzed using a GC-MASS spectrometer (Shimadzu-LKB 9000, column: 3% SE-52 on chromosorb W, 2 m, temp 195° C).

Extraction and Purification of the Mistletoe OMT Mistletoe young shoots (815 g) were homogenized by a Ultratrax homogenizer in the presence of polyclar AT (15 g) and 0.25 M K-phosphate buffer (pH 7.4; 1540 ml) containing 1 mM of EDTA, 3 mM of 2-mercaptoethanol and 5 mM of Na-ascorbate in an ice basket. The homogenate was filtered with three-layered cheese cloth and the crude juice obtained (1640 ml) was centrifuged at 17000 g for 30 minutes (supernatant 1585 ml). After dialysis of the supernatant against 0.025 M K-phosphate buffer (pH 7.5) containing 3 mM of 2-mercaptoethanol (5 l) for 10 hours x 2 times, the dialyzate was treated with DEAE-cellulose (50 g), which was previously bufferized with the 0.025 M K-phosphate, for 10 hours. The absorbed fraction was eluted by the 0.025 M K-phosphate buffer containing 0.25 M KCl and then the eluate (1060 ml) containing 3 mM of EDTA was precipitated by addition of solid ammonium sulfate (547 g) adjusted pH 7.2-7.4 with 1% NH_3 aq. solution. The precipitate was collected by centrifugation at 5600 g for 30 minutes and dissolved in 0.5 saturated

ammonium sulfate solution (100 ml). The undissolved fraction was collected by the same way and the precipitate was dissolved in 15 ml of the 0.025 M K-phosphate buffer (16.4 ml). The solution was applied to a Sephadex G100 column (2.7 x 100 cm, up-ward flow rate 10.7 ml/hour)which was equilibrated with the 0.025 M K-phosphate buffer and the active fraction was collected (146 ml). Protein contents were determined by the method of Lowry et al.[82]

Tracer Experiment and Nitrobenzene Oxidation Phenylalnine-U-^{14}C(6.67 μCi, 24.2 μmole) was fed to a young shoot (23.4 g) of mistletoe and metabolized for 24 hours. After removal of the leaves (12 g) the shoot was homogenized with hot 80% of EtOH using a Waring blendor and the residue was extracted with EtOH/benzene (1:2) for 9 hours. The sample thus obtained was oxidized by nitrobenzene oxidation: sample (3 g) was added in a mixture of 2 N NaOH (60 ml) and nitrobenzene (4.2 ml) and kept at 170° C for 2 hours in a rotating autoclave. The aldehyde fraction was extracted by a conventional method and each aldehyde was separated on preparative TLC (solvent: water saturated iso-propylether). These aldehydes were converted to m-nitrobenzoylhydrazone derivatives (each aldehyde 15 mg was dissolved in water 4 ml and 5% solution of the hydrazide 3 ml was added) which were recrystallized twice from water.

OMT, Phenylalanine ammonia-lyase and Cinnamate-4-hydroxyalse Assay Sliced tissue of the mistletoe was incubated under

illumination and 5 g portion of the tissue was homogenized every 6 hours successively with the presence of 0.2 M Tris-HCl buffer (pH 7.5, 5 ml) containing 3 mM 2-mercaptoethanol, sea sand (2.5 g) and polyclar AT (0.7 g) in a cold mortar. Then, the homogenate was squeezed by cheese cloth and centrifuged at 17000 g for 10 minutes. The supernatant was used for OMT assay by the procedure described in chapter II. Rf values of the products formed were found to be identical with those of authentic compounds by paper chromatoscanning (solvent: toluene-HOAc-H_2O 4:1:5, organic layer). On the other hand, the supernatant was treated with Dowex 1 x 4 (1 g) for 40 minutes in an ice cold bath. After centrifugation (at 1500 g for 10 minutes), the supernatant thus obtained was assayed for PAL activity by a conventional method.[135] Cinnamate-4-hydroxylase was extracted by the same procedure as the case of PAL except that the tissue was incubated for 20 hours in K-phosphate buffer. The reaction mixture for the enzyme assay contained cinnamate (4.4 μmole), NADPH (2 μmole), glucose-6-phosphate (5 μmole), K-phosphate (150 μmole), 2-mercaptoethanol (3 μmole) and enzyme solution (0.5 g fresh weight), in 3 ml of total volume. The product which was purified by preparative TLC (twice, solvent $CHCl_3$-HOAc-H_2O 4:1:1) was found to be identical with the authentic compound by mass spectrometry (Shimadzu-LKB 9000).

TABLE 11 CHEMICAL PROPERTIES OF MISTLETOE LIGNIN

		Viscum	*Celtis*
LIGNIN CONTENTS		22.0 %	21.6 %
EMPERICAL FORMULAE OF MWL*		$C_9H_{5.77}O_2(H_2O)_{1.44}(OCH_3)_{1.33}$	$C_9H_{5.44}O_2(H_2O)_{1.45}(OCH_3)_{1.29}$
UV	λ_{MAX} (NM)	277.5	278.0
	A (l/g-cm)	15.3	14.4
IR		TYPICAL ANGIOSPERM TYPE	
^{13}C-NMR		TYPICAL ANGIOSPERM TYPE	
NITROBENZENE OXIDATION**		1.7	—
ACIDOLYSIS***		0.92	0.73

* UPPER: *Viscum*, LOWER: *Celtis*

** THE RETIO, SYRINGALDEHYDE/VANILLIN

*** THE RATIO, SYRINGYL ACETONE/GUAIACYL ACETONE

TABLE 12 RECOVERY OF MISTLETOE FA-ACTIVITY DURING THE PURIFICATION

PURIFICATION PROCEDURE*	PROTEIN (%)	RECOVERY (%) FA	RECOVERY (%) SA	FOLD FA	FOLD SA	SA/FA RATIO
STEP 1	100.0	100.0	100.0	1.0	1.0	46.7
STEP 2	71.8	85.9	92.0	1.2	1.3	50.0
STEP 3	10.8	1019.8	56.9	94.4	5.3	2.6
STEP 4	7.5	1055.6	51.2	141.0	6.9	2.3
STEP 5	3.8	943.8	44.1	246.5	11.5	2.2

* STEP 1: CRUDE HOMOGENATE, STEP 2: CENTRIFUGATION, STEP 3: ION-EXCHANGE RESIN TREATMENT, STEP 4: AMMONIUM SULFATE FRACTIONATION, STEP 5: GEL FILTRATION CHROMATOGRAPHY

TABLE 13 INCORPORATION OF L-PHENYLALANINE-U-^{14}C INTO MISTLETOE LIGNIN*

NITROBENZENE OXIDATION PRODUCTS‡	YIELD % OF KL#	(RATIO)	SPECIFIC ACTIVITY CPM / μMOLE	DILUTION VALUE
P-HYDROXYBENZALDEHYDE	3.0	(0.7)	570.1	1070.9
VANILLIN	4.4	(1.0)	1583.8	385.5
SYRINGALDEHYDE	8.1	(1.7)	1090.9	559.6

* VISCUM ALBUM 24.5 G (SHOOT 13.4 G): L-PHENYLALANINE 6.67 μCi / 24.2 μMOLE

‡ *m*-NITROBENZHYDRAZONE DERIVATIVES # KLASON LIGNIN

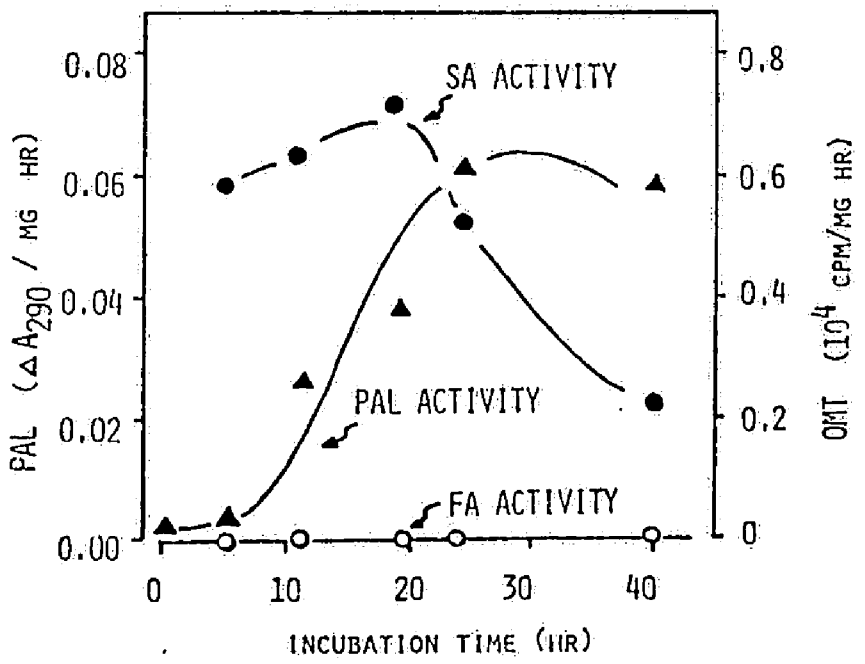

FIGURE 9 DEVELOPMENT OF ENZYME ACTIVITIES DURING THE INCUBATION OF MISTLETOE TISSUES

Chapter IV Roles of Bamboo O-Methyltransferase in Grass Lignin.

INTRODUCTION

The O-methyltransferases (OMTs) involved in lignin biosynthesis could be roughly classified into gymnosperm- and angiosperm-types.[61] The former catalyzes the formation of guaiacyl-unit but scarcely does that of syringyl-unit. The purified pine OMT was discussed in chapter I. On the other hand, the latter type OMT catalyzes both guaiacyl- and syringyl-unit formations. The purified aspen and mistletoe OMTs were discussed in respective chapters II and III.

The formation ratio of sinapate (SA) to ferulate (FA) by the enzyme, i.e. SA/FA ratio, is relatively correlated with the raito of syringaldehyde to vanillin (S/V), which are lignin degradation products by alkaline nitrobenzene oxidation. The SA/FA ratio more directly reflects genetic informations comparing with the S/V ratio, thus being useful for phylogenic plant classification.[61] The details will be discussed in the following chapter.

In lignin biosynthetic pathway, ferulate-5-hydroxylase has not been isolated and the distribution of sinapoyl:CoA ligase in angiosperms has been reported to be not universal. The situation indicates that the biosynthetic pathway for syringyl lignin is not conclusively elucidated and is making doubt on general SA-participation in syringyl lignin biosythesis.

However, Gramineae is considered to utilize SA as an inter-

mediate, because of the presence of sinapoyl:CoA ligase,[51] efficient incorporation of sinapate into lignins,[1,3] and universal distribution of free and esterified sinapate in Gramineae. It seems, therefore, that the formation of syringyl to guaiacyl lignin is under control of the SA/FA ratio in bamboo. Bamboo OMT which belongs to angiosperm-type catalyzes the formation of both guaiacyl- and syringyl-units at nearly same rates.[61,84,96,136] This chapter forcuses on the control mechanism of the both unit formations by bamboo OMT.

RESULTS

General Properties of Bamboo OMT

Bamboo OMT was extracted from Phyllostachys pubescens young shoots, which showed specific activity of ca. 3 pKat per mg protein for FA-activity. The properties of OMT in crude preparation is summarized as follows. The crude enzyme showed optimal pH ca. 8.0 for both FA- and SA-activities with half maximal activities at pH 8.6 ± 0.2 and 6.4 ± 0.2 (see also in ref. 96). PCMB (3 mM) and EDTA (10 mM) moderately inhibited the enzyme activity (10% and 20% inhibition, respectively), but monoiodoacetate (0.3 and 3 mM) showed no significant inhibition.[96] EDTA and SH reagents prevented its deactivation when the enzyme was precipitated by ammonium sulfate.[96] Mg^{2+} ion and SH group seem to be involved in the enzymic methylation.[96] In addition, purified OMT was found to be an acidic protein with an isoelectric point at pH 4.61 at 4° C as shown in Figures 12 and 13.

The Ratio SA- to FA-activities in the Crude Preparation

The ratio SA- to FA-activities were found to be somewhat varied, when the crude OMT was assayed in different hydrogen ion concentrations. The fractions by ammonium sulfate precipitation also showed the ratio-fluctuation. Thus, the crude bamboo OMT concentrated by ammonium sulfate precipita-

g.10 (p. 83)
g.11 (p. 83)

tion was preliminary examined by chromatography on the OMT-polymorphism. Figure 10 and 11 showed the profile of crude enzyme on DEAE-cellulose and Sephadex G200 chromatography, where neither OMT-polymorphism nor dissolution of the FA- and SA-activities were observed. The apparent SA/FA ratio tends to decrease from near the OMT peak to its base in the chromatograms.

Partial Purification of Bamboo OMT

le 14 (p. 82)

Bamboo OMT was examined by purification if FA- and SA-activities are resolved. Table 14 summarizes two series of purification achieved. Bamboo OMT was rather stable, in contrast to the enzymes of aspen cambial zone and developing xylem (chapter II). Ammonium sulfate fraction at step 2, precipitated by 55%- to 20%-saturated ammonium sulfate, contained ca. 90% of the OMT activity. Elution profiles of the chromatograms during the purification were almost identical to the crude ones. The bamboo OMT was finally purified 97-fold with 5% recovery, specific activities of which were 2.50 and 3.05 nKat per mg protein for FA- and SA-activities, respectively. The ratio SA/FA kept constant during the purification, and was found to be 1.25 $\pm$ 0.1 at pH 8.0. The final preparation was further examined by electrophoresis and isoelectric forcusing, if the FA- and SA-activities were resolved, and/or

g. 12 (p. 84)
g. 13 (p. 84)

showing multiple forms. FA- and SA-activities showed a single peak at the same place as shown in Figures 12 and 13 and these accurate methods conclusively demonstrated no resolution of the both activities.

Thermo-stability of the Bamboo OMT

. 14 (p. 85)

FA- and SA-activities were similarly decreased by 10 minute-incubation at various temperatures (Figure 14C and 14D) The both deactivation-time courses were almost identical at 50° C, but with a small difference at 45° C in both presence and absence of the phenolic substrates (Figure 14A and 14B

le 15 (p. 82)

Table 15). The heat treatment at 45°C changed SA/FA ratio which was higher in the presence of substrates than in the absence (Table 15). The half-life period at 50° C was found to be six times longer in the presence of substrates than in the absence as shown in Figure 14A and 14B. Protecting effect of 5-hydroxyferulate on the heat treatment was remarkable comparing with that of caffeate although the former substrate was not effective on the enzyme stabilization when incubated at 4° C for 5 days. The OMT-stability at 4° C was improved by adding glycerol which kept 96% of the activity during the 5-day-incubation at 4° C, while no glycerol kept 74% of the activity during the incubation.

Substrate Specificities of Bamboo OMT

Methylation by bamboo OMT was found in caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or little methylation in chlorogenate, isoferulate, m-, p-coumarate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxyphenylmanderate, gallate, pyrocatechol, pyrocatechol phthalein and d-catechin. Km values for caffeate and 5-hydroxyferulate were 5 X 10^{-5} and 10^{-5} M, respectively, in excess S-adenosyl-L-methionine. Figure 15 shows that the latter phenolic substrate competitively inhibited FA-formation, for which Ki was identical to the Km for the same substrate.

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p. 86)

DISCUSSION

Bamboo OMT is chromatographically demonstrated to be a single enzyme which catalyzes FA- and SA-formations with preferential latter formation. This is supported by remaining a constant SA/FA ratio during the purification, and by deactivation pattern on heat treatment. The finding that caffeate and 5-hydroxyferulate were competitively methylated also suggests that the bamboo OMT is a single enzyme which has one active methylating-center for FA- and SA-formation. Although apparent polymorphism had been reported on a previous work,[136] no OMT-polymorphism was observed in the present experiments.

Some variation of SA/FA ratio was observed in the crude enzyme preparation possibly due to the following reasons. When the OMT-activity decreases to a control value, the SA/FA ratio gradually decreases and comes close to one, and such a low activity causes experimental error, resulting in the ratio-flactuation. Another reason is due to low protein concentration. When crude preparation contains small amounts of protein (less than ca. 80 ng), the apparent ratio was also varied (data not shown).

The facts described above partially accounts for the variation, but the flactuation of the ratio may also occur by partial denaturation of the enzyme by heat, acid or alkali,

and ammonium sulfate, causing its conformational change. It is concievable that such modification which may be derived by the cytoplasmic alterations *in vivo*, temperature, pH changes, and protease attack etc., may cause the variation of SA/FA ratio. In other words, OMT might finely change its substrate specificity via cytoplasmic alterations to adjust guaiacyl- and syringyl-formations in lignin biosynthesis.

The kinetic study demonstrated that both FA- and SA-formations were competitively controlled, suggesting that *in vivo*, a feedback inhibition might operate on the former formation by the latter substrate.

Two multiple forms were found in soybean p-coumaroyl:CoA ligase[48] and cinnamyl alcohol dehydrogenase,[55] which respectively correspond to catalyze only guaiacyl group or both guaiacyl and syringyl groups. Syringyl lignin increases during xylem differentiation in angiosperm,[88,97] which seems to be under control by the enzymes involved in the reduction of sinapate to sinapyl alcohol.[88] Induction of the enzyme is regarded as a coarse adjustment in the biosynthesis of syringyl lignin. Because syringyl lignin gradually increases during the tissue differentiation, the adjustment take rather long period of time. While the bamboo OMT seems to regulate lignin biosyntesis by means of fine adjustment, i.e. the conformational change and the feedback inhibition as described above, which are capable of very minute alterations.

Gymnosperm OMT is not able to catalyze SA-formation (SA/FA ratio ca. 0.1), while angiosperm OMT catalyzes both FA- and SA-formations (SA/FA ratio ca. 3). The finding that the SA/FA ratio showed ca. one in bamboo OMT seems to be characteristic for Gramineae and allied species, e.g. Oryza sativa 0.9, Triticum aestivum 1.0, Zisania latifolia 1.1, Sparganium stoloniferum 1.5. Considering the ratio of syringaldehyde to vanillin, produced by nitrobenzene oxidation of lignin, Gramineae OMT seems to form another group.

The SA/FA ratio is found to be one of the important step in the formation of guaiacyl and syringyl lignin and could be used as a marker for phylogenic evolution in plants.

EXPERIMENTAL

Materials

The buffers used were the following. Buffer A: 0.2M K-phosphate buffer (pH 8.0), containing each 5 mM of 2-mercaptoethanol (ME), CySH, NaN_3 and isoascorbate. Buffer B: 0.5M K-phosphate (pH8.0). Buffer C: 0.02M K-phosphate buffer (pH7.4), containing each 5 mM of CySH and ME. Scintillator for radioactive counting contains toluene - 2,5-diphenyoxazole-1,4-bis-[2-(4-methyl-5-phenyloxazole-2-yl]benzene (1.0_l : 4_g : 0.1_g). S-Adenosyl-L-methionine-^{14}C (SAM; specific activity 53.1 μCi/μmole) was purchased from New England Nuclear and diluted with cold one for the enzyme assay. Ampholine Carrier Ampholytes (LKB-Produkter AB; 40% w/w, pH 3 - 6) was obtained from American Commercial Co.Ltd., Japan.

OMT Assay

Standard reaction mixture (total volume 1.0 ml) contains: Buffer B (50 μmole), CySH (10 μmole), ME (10 μmole), isoascorbate (0.5 μmole), enzyme preparation (less than 10^4 cpm), SAM (0.05 μCi/0.5 μmole), and phenolic substrate (0.5 μmole). The reaction mixture without SAM was preincubated for 5 min., then the SAM was added as a starter and incubated for 0.5 - 1.0 hr. at 30° C. The reaction was terminated by the addition of 5% of HCl (0.5 ml), and the products formed were extracted with ether. The extracts (first 10 ml, then 5 ml of ether) were

combined and evaporated under reduced pressure. The residues were dissolved in dioxane (0.5 ml) and transfered into a vial containing the scintillator (5 ml), then counted by a Beckmann LS100 scintillation counter. The counting efficiency was 89%, and 10^4 cpm corresponded to 50.6 nmole of the methylated products formed.

Extraction of Bamboo OMT

Bamboo (Phyllostachys pubescens) young shoots were harvested in June, from which enzymes were instantly extracted in a cold room (4 - 6° C) as follows. The sliced shoots (6 kg) were homogenized with buffer A (4 l) by a domestic mixer, then the homogenate was squeezed with four-layered guaze. The juice obtained were centrifuged at 10^4 rpm for 20 min., and supernatant of which was precipitated by adding solid ammonium sulfate (finally 70%-saturation). The precipitate was stored at -20° C until for use.

Purification of bamboo OMT

Step 1: The stock preparation (33 ml) was centrifuged at 10^4rpm for 15 min.

Step 2: The precipitate was dissolved in 55%-saturated ammonium sulfate-buffer C soln. (30 ml) with EDTA (150 μmole), and centrifuged at 10^4 rpm for 15 min. Then, the same procedure was repeated for the precipitate. The precipitate thus obtained was dissolved in 20%-saturated ammoium sulfate-buffer C soln. (20 ml) which contained EDTA (100 μmole) and

5-hydroxyferulate (330 μmole) and centrifuged at 10^4 rpm for 15 min. The precipitate was again subjected to the same procedure. Subsequently, EDTA (250 μmole) and solid ammonium sulfate (17 g; finally 70%-saturation) were added to the supernatant and the precipitate was collected by centrifugation. The precipitate was then passed through a Sephadex G25 column (40 cm X 2.8 cm; V_o = 125 ml).

Step 3: DEAE-cellulose (6 g of dry weight, which was preliminary washed and bufferized with buffer C, was added to the enzyme soln. desalted (80 ml). The soln. was stirred for 20 min., and then the enzyme absorbed on the ion-exchange-cellulose was charged on a column (6 cmφ). After washing it with buffer C (150 ml) and subsequently 0.05M KCl-buffer C soln. (235 ml), the fraction eluted with 0.2 and 0.3 M KCl-buffer C were collected (182 ml).

Step 4: The combined fraction was precipitated by adding ammonium sulfate (finally 60%-saturation) and it was centrifuged at 10^4 rpm for 15 min. The precipitate was then dissolved in buffer C, and applied on a Sephadex G200 column (flow rate 12 ml/hr., V_o = 170 ml, solvent for the elution: buffer C). The fraction numbers from 32 to 54 were collected (71.5 ml) for further purification.

Step 5: The preparation was applied on a DEAE-cellulose column (bufferized with buffer C, 10 cm X 1.7 cmφ). Then, the enzyme was successively fractionated by stepwise elution

with following buffer solns: buffer C, 0.1 M-, 0.15 M-, and 0.25 M-buffer C soln. The fraction eluted by 0.15 M was collected, which was final preparation.

The pH was maintained by adding 1 N NH_3 soln, during the ammonium sulfate precipitation. The second purification was almost same as the procedure described above, except that step 5 was operated using a linear gradient elution.

Disc Electrophoresis

Disc electrophoresis was carried out by the method of Davis.[15] The gel after electrophoresis, was directly assayed for FA- and SA-activities.

Isoelectric Fractionation

According to the method of Vesterberg et al.,[137] carrier Ampholyte was charged on an isoelectric forcusing-column (LKB-ampholine, 110 ml), where the final Ampholytes concentration was 1%. After charging sample (Step 5), a voltage (700 V; initial current ca. 8 mA) was applied for 36 hr. at 4° C.

Protein Determination

The sample for protein determination was dialyzed against buffer C. Subsequently, coloration with Lowry's phenol reagent was followed and protein was determined at 750 nm.[82] Protein contents were calculated by means of serum albumine-calibration curve. The net contents were obtained by subtracting the absorbance of the buffer C as a control.

TABLE 14 PURIFICATION OF BAMBOO OMT

PURIFICATION PROCEDURE	TOTAL PROTEIN (MG)	TOTAL ACTIVITY (UNITS)		SPECIFIC ACTIVITY (UNITS/MG)		RECOVERY (%)		SA/FA RATIO	FOLD
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	1.590	2.600	3.190	1.6	2.0	100	100	1.22	1
2. 20-55% "	695	2.360	2.980	3.4	4.3	91	93	1.26	2
3. DEAE-CELLULOSE	92	1.210	1.630	13.2	17.7	47	51	1.34	9
4. SEPHADEX G-100	13	396	520	30.0	39.5	15	16	1.31	20
5. DEAE-CELLULOSE	1.4	165	195	117.0	138.0	6	6	1.18	69

PURIFICATION PROCEDURE	TOTAL PROTEIN (MG)	TOTAL ACTIVITY (UNITS)		SPECIFIC ACTIVITY (UNITS/MG)		RECOVERY (%)		SA/FA RATIO	FOLD
		FA	SA	FA	SA	FA	SA		
1. 0-70% Am_2SO_4	2 900	5 980	7 300	2.06	2.52	100	100	1.22	1
2. 20-55% "	1 300	6 890	8 640	5.3	6.8	118	118	1.26	3
3. DEAE-CELLULOSE	118	5 480	6 870	46.5	58.3	92	93	1.25	23
4. SEPHADEX-G 200	65	3 720	4 180	57.5	64.0	63	57	1.11	28
5. DEAE-CELLULOSE	1.5	300	368	200.0	244.0	5	5	1.22	97

TABLE 15 THERMOSTABILITY OF BAMBOO OMT AT 45° C IN THE PRESENCE OR ABSENCE OF PHENOLIC SUBSTRATES

TIME	MINUS SUBSTRATES (CPM)			PLUS SUBSTRATES (CPM)		
MIN.	FA	SA	SA/FA	FA	SA	SA/FA
0	22630	31530	1.39	25130	30930	1.23
10	10510	13310	1.27	13510	18010	1.33
20	7300	11430	1.57	9530	19030	2.00
30	8030	8630	1.07	10830	16030	1.48
40	8340	9640	1.16	11240	17940	1.60
50	7170	8570	1.20	10670	15870	1.49
100	4090	5090	1.24	6190	7390	1.19

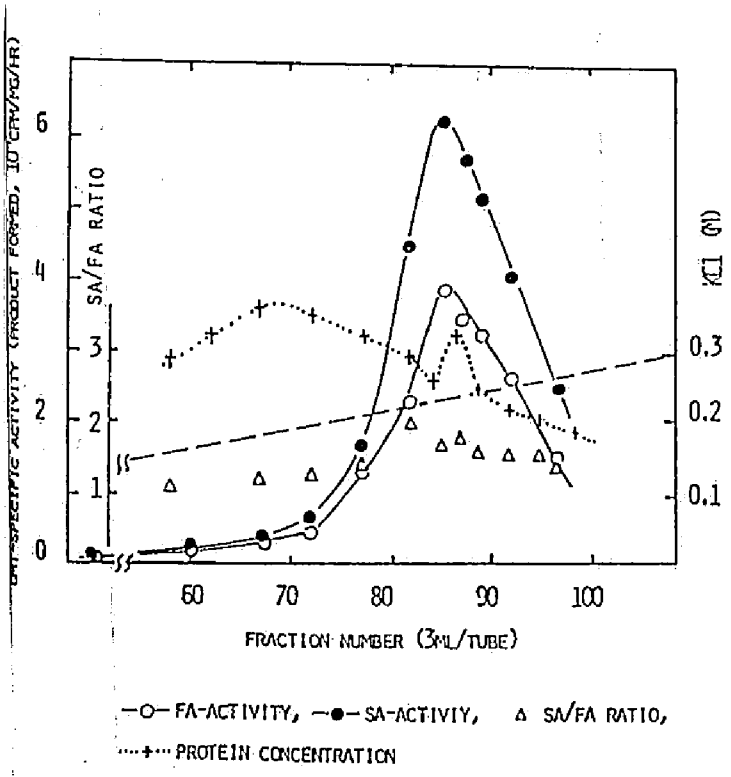

FIGURE 10 ELUTION PATTERN OF BAMBOO OMT ON A DEAE-CELLULOSE COLUMN

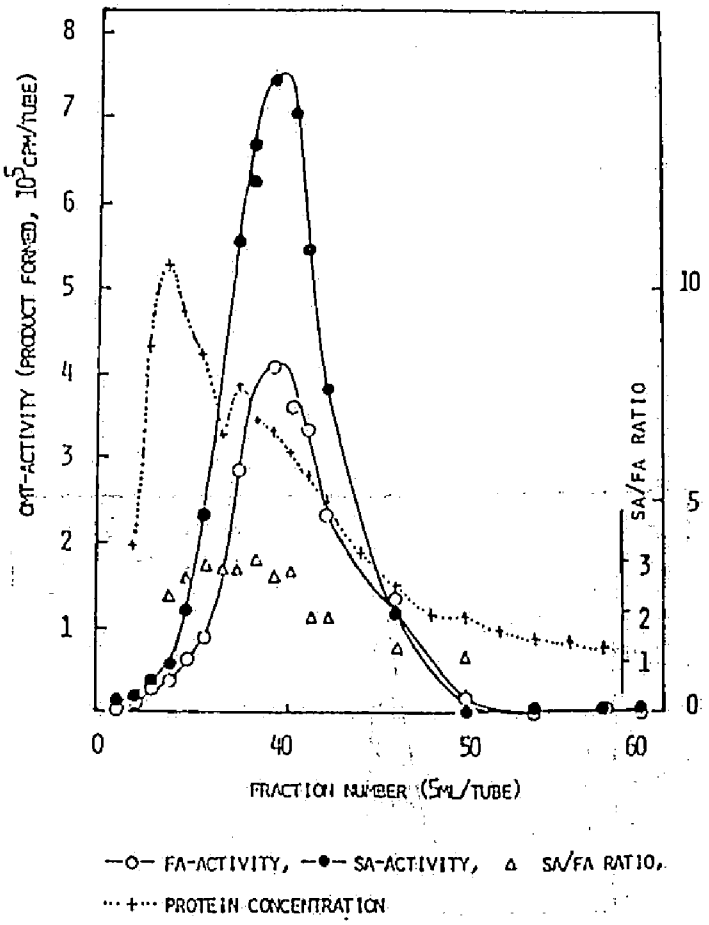

FIGURE 11 GEL-FILTRATION PATTERN OF BAMBOO OMT ON SEPHADEX G100

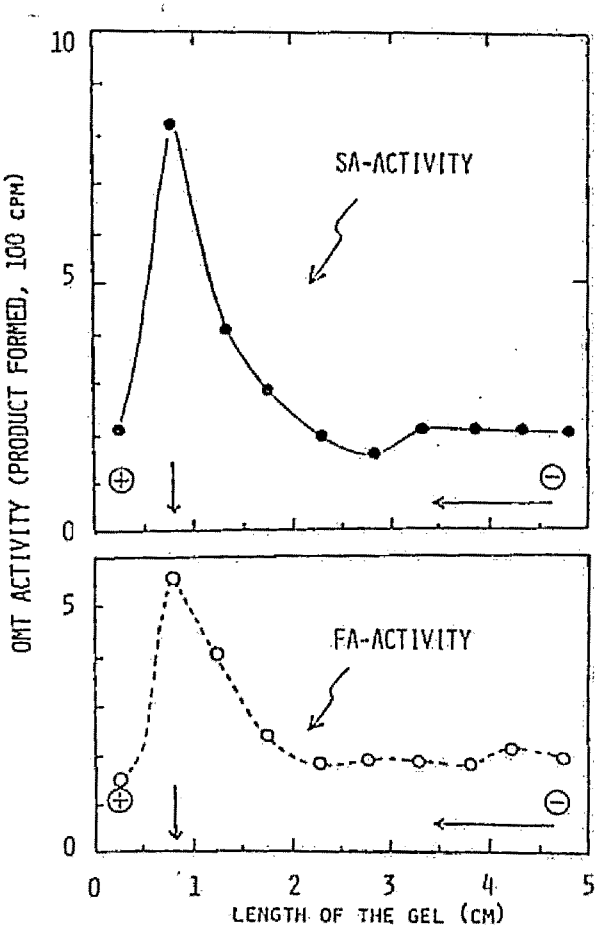

FIGURE 12 ELECTROPHORETIC PATTERN OF BAMBOO OMT ON POLYACRYLAMIDE GEL

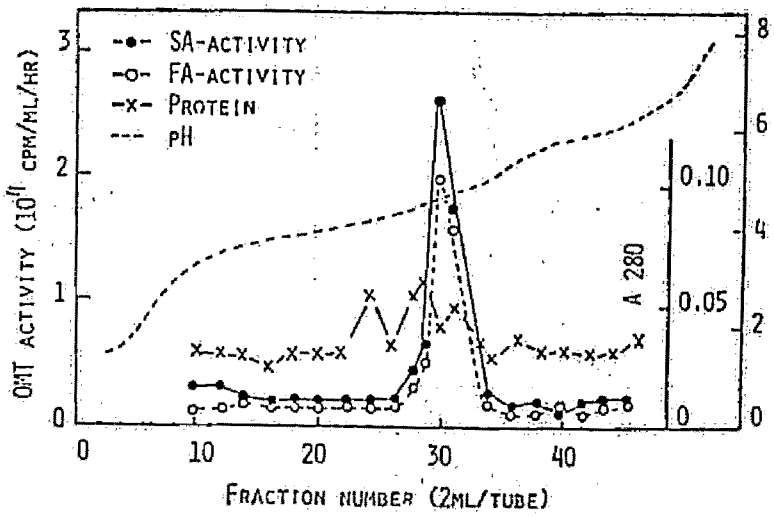

FIGURE 13 ISOELECTRIC FOCUSING PATTERN OF BAMBOO OMT

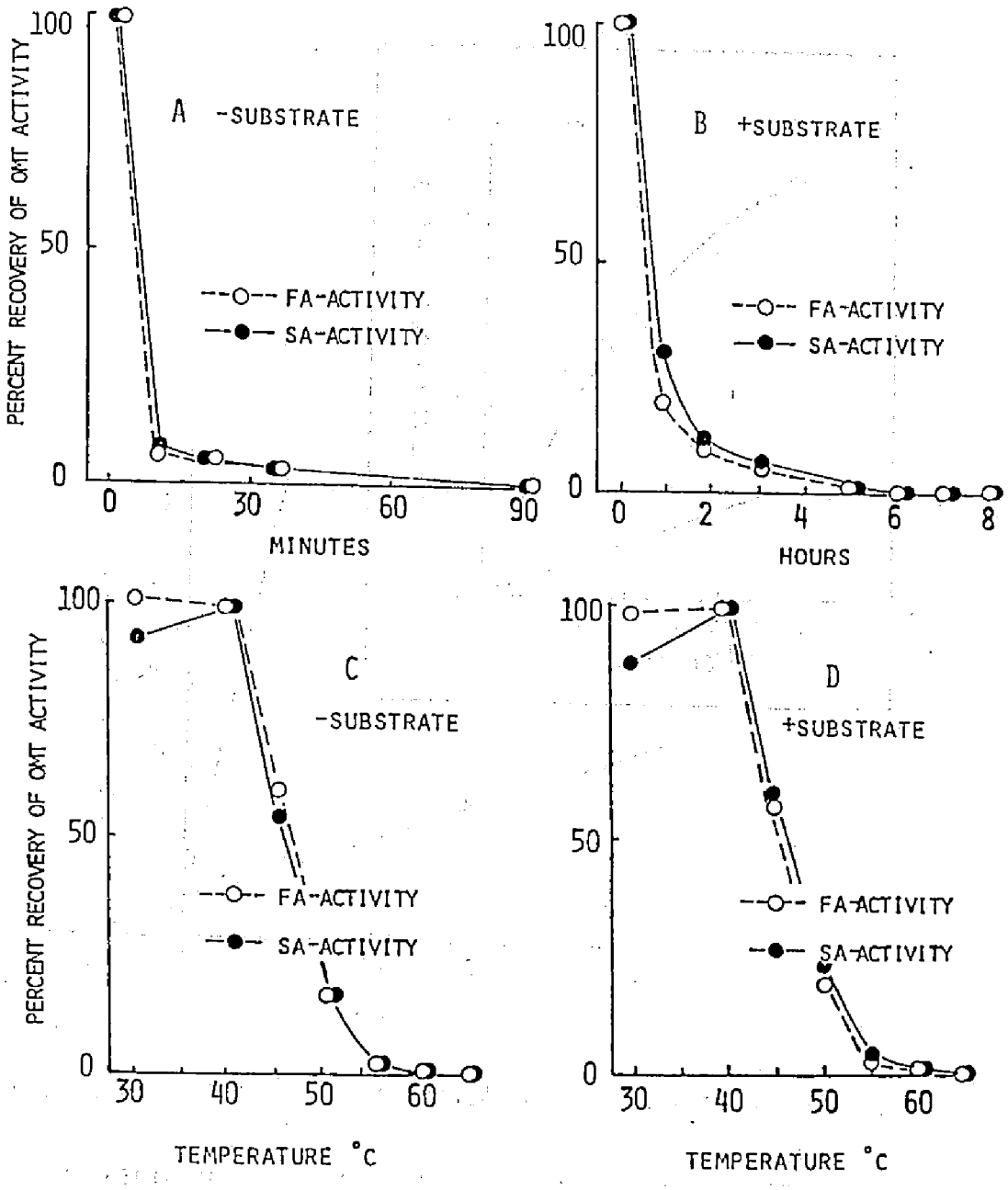

FIGURE 14 HEAT TREATMENT OF BAMBOO OMT

A & B: EFFECT OF CAFFEATE OR 5-HYDROXYFERULATE ON THE THERMO-STABILITY OF BAMBOO OMT DURING HEAT TREATMENT AT 50° C.

C & D: DENATURATION PATTERNS OF BAMBOO OMT ON 10 MINUTES HEAT TREATMENT IN THE PRESENCE AND ABSENCE OF CAFFEATE AND 5-HYDROXYFERULATE.

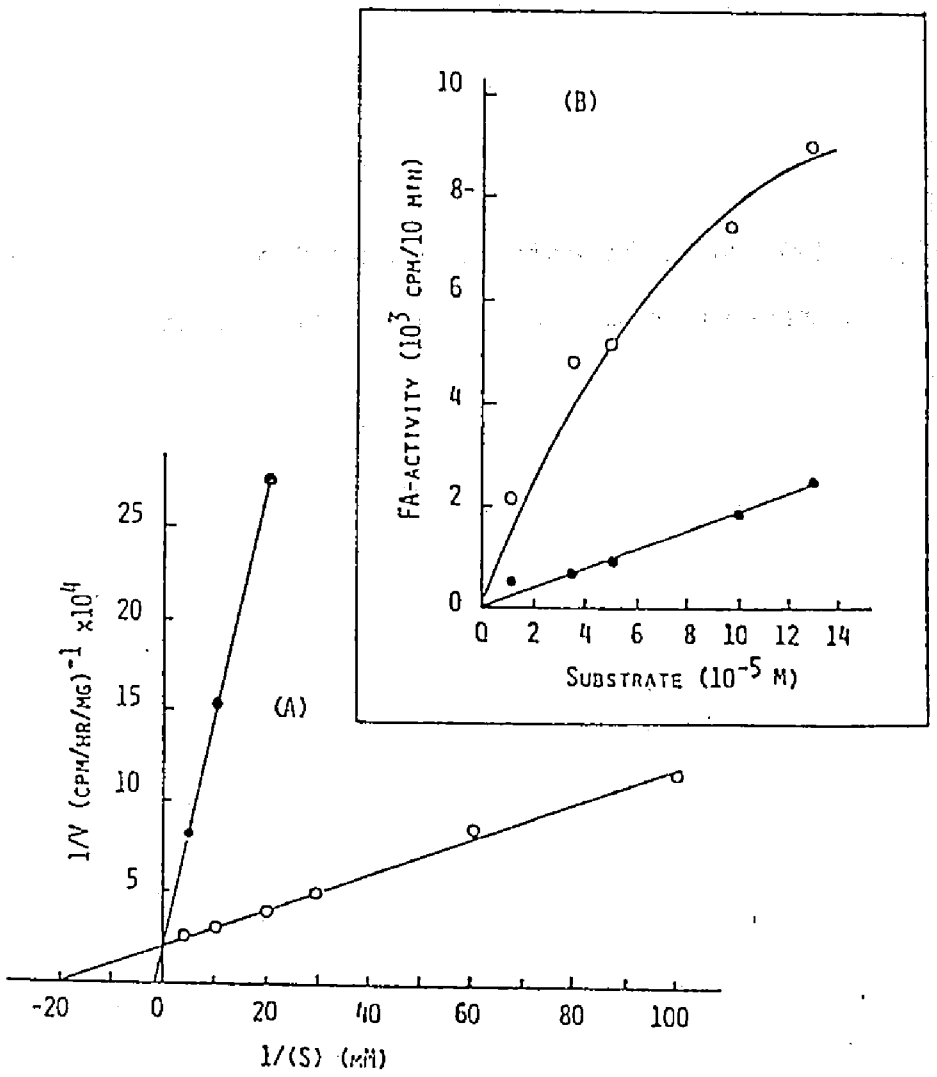

FIGURE 15 COMPETITIVE INHIBITION OF FA-ACTIVITY BY 5-HYDROXY-FERULATE

A: RECIPROCAL PLOT OF METHYLATION-VELOCITY AGAINST CAFFEATE CONCENTRATION IN THE PRESENCE AND ABSENCE OF 5-HYDROXYFERULATE (10^{-4}M), 70% AMMONIUM SULFATE PPT, PASSED THROUGH SEPHADEX G100

B: EFFECT OF 5-HYDROXYFERULATE (4×10^{-5} M) ON THE METHYLATION-VELOCITY FOR CAFFEATE, (32-FOLD PURIFIED ENZYME WAS USED).

Chapter V Comparative Evaluation of Plant O-Methyltransferases in Lignin Biosynthesis.

INTRODUCTION

Mäule color reaction has been generally used as a tool to distinguish syringyl lignin from guaiacyl lignin in angiosperm and gymnosperm.[8-10] Many workers followed it up mainly by surveying lignin degradation products.[7,11,64,138-141] For example, alkaline nitrobenzene oxidation has been frequently applied and the aromatic aldehydes yielded were analyzed.[7,8,11] Gymnosperm lignins almost entirely yield vanillin, but angiosperm ones generally produce vanillin (V) and syringaldehyde (S) in the nitrobenzene oxidation. Grass lignins yield p-hydroxybenzaldehyde in addition to the both aldehydes. These aldehydes show the lignin structural units corresponding to guaiacyl, syringyl and p-hydroxyphenyl nuclei, and they are considered to be related to lignin evolution.[7,8,11,64,138-141]

A few O-methyltransferases (OMTs) have been characterized and purified,[67,68,75,76,84,87,91,99,142,143] and it was found that the substrate specificity is closely related to the corresponding plant classes. Gymnosperm OMTs only catalyzing feruralte- (FA) formation (chapter I), while angiosperm ones catalyzing both FA- and sinapate (SA)-formation[61] (chapter II-IV). Thus, the formation ratio of SA to FA might be a good indicator to evaluate lignin evolution. The SA/FA ratio more directly reflects genetic information comparing to the results obtained by chemical anaylses of lignins, e.g. S/V ratio.

Another advantage is that the ratio essentially keeps constant during plant differentiation, which is ascribed to the fact that an OMT carries both FA- and SA-activities, the ratio of which shows a definite value.[84,143] This chapter will be discussed on plant OMTs if the SA/FA ratios are generally available to investigate lignin evolution.

RESULTS AND DISCUSSION

General Remarks

The plants surveyed are listed in Table 16-18. OMTs were found to be distributed widely in the plants. The tables show SA/FA ratio, values of which were listed only when the enzyme activities were more than 10^3 cpm/hr. Enzyme activities in such levels were difficult to detect especially in Pteridophyta and gymnosperm (Table 16). The difficulty might be ascribed to high terpene contents, and/or presence of some inhibitors in the tissues, although the young shoots were carefully selected and homogenized with additives to prevent such trouble. The low enzyme levels are also partially due to the routine procedure, condition of which is not always optimal for the enzyme extraction and assay.

Generally the SA/FA ratio in various plants showed a good contrast between gymnosperm and angiosperm. As postulated, gymnosperm OMTs catalyze almost only FA-formation (Table 16), while angiosperm ones do both FA- and SA-formation (Table 17 and 18). This well explains why gymnosperms almost entirely contain guaiacyl unit and angiosperms contain both guaiacyl and syringyl units in lignins.

Gymnosperm OMTs

Most of the gymnosperm OMTs, as represented by the Pinaceae

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enzymes, showed low SA/FA ratios with a few exceptions (Table 16). Thuja, Podocarpus and Ephedra OMTs showed rather high ratios in this experiment, although the latter two were not able to confirm because of the low enzyme activity. The ratio in Thuja orientalis seedlings was found to be 0.83, the value of which was discussed in relation to lignan biosynthesis in Thuja.[68] However, the ratio might be also evaluated from another point of view.

Most of the species in Cupressaceae contain only guaiacyl lignin except Tetraclinis which contains guaiacyl-syringyl lignin.[7,8,64] In addition to this fact, the finding of a high SA/FA ratio in Thuja indicates that the lignin biosynthetic pathway might be tinged with characteristics of angiosperm type in Cupressaceae. The ratio SA/FA more directly reflects genetic codes comparing with lignins as a phenotype, and the ratios seem to show a genetic variation in this family. Thus, the ratio might be a good tool in order to analyze evolution stage of such species in Cupressaceae. The situation is probably the same in Podocarpaceae and Cycadaceae, because some species in the families contain both guaiacyl-syringyl lignin in spite of gymnosperm.[7,8] These groups are considered to be transition type between gymnosperm and angiosperm in the point of lignin biosynthesis. On the other hand, the lignin biosynthetic pathway in Gnetales is considered to be completely transformed into angiosperm one, because they

contain guaiacyl-syringyl lignin.[7,8]

Angiosperm OMTs

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Polycarpiidae and Amentifloriidae gave normal ratio (2.0-3.5; Table 17) in this experiment. These groups are considered to be primitive angiosperm in the viewpoint of polyphyletic theories in angiosperm phylogeny.[16] Winteraceae, which could not be surveyed, belongs to vessel-less Polycarpiidae, some of which give low S/V ratios.[7,8] Trochodendron OMT, however, showed rather a low SA/FA ratio, the plant of which lacks vessel elements but contains guaiacyl-syringyl lignin.[7,11,64] It is interesting to note that the primitive plant OMT gives the ratio inbetween gymnosperm and angiosperm.

Usual ratios were observed in a few plants. Crude mistletoe (Viscum album) OMT apparently showed a very high ratio, but the ratio by the purified enzyme was found to be normal (SA/FA = 2.2).[99] The finding that FA-activity was almost completely inhibited in crude mistletoe preparation, strongly suggests the presence of OMT-inhibitor in this plant. Another interesting example was Erythrina, secondary xylem except fibers of which contains almost entirely guaiacyl lignin, although the ratio SA/FA was normal angiosperm type.[98] Ferulate-5-hydroxylase was presumed to be absent in the plant, which may result in low syringyl units.

Herbaceous angiosperm lignins contain rather small amounts

of methoxyl group per C_9 unit, comparing with that of normal woody angiosperm lignins.[64] For example, swede (Brassica napobrassica) roots the lignin of which comprise almost entirely guaiacyl unit showed a low SA-activity.[87] The low SA-activity might be resulted from regressive evolution of the OMT because OMTs are usually considered to give a constant ratio during differentiation. The low ratio might be also ascribed to the poor vascular bundle tissues in the swede roots, lignin contents of which are usually very low. In such tissue as described above, the OMT activity which directly involved in lignin biosythesis might be low comparing with other OMT levels, e.g. flavonoid specific one.[76] When such OMT coexists in the herbaceous tissues surveyed, the apparent SA/FA ratio will be affected. For instance, a flavonoid specific OMT in soybean (Glycine max) suspension cells showed ca. 1.4 of the ratio,[76] while a lignin specific OMT in the same cells showed ca. 1.9 of the ratio.[75] If the lignin specific OMT presents in low level, apparent ratio would affected by such ratio of the flavonoid specific OMT.

Gramineae OMTs showed a SA/FA ratio around 1.0 and other OMTs in monocotyledon showed rather a small ratio comparing with normal angiosperm OMTs (Table 18). The SA/FA ratio in crude bamboo (Phyllostachys pubescens) OMT was demonstrated to be the lignin specific one and no other OMTs were detected in the crude preparation.[84,143] In other words, crude bamboo

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OMT showed a net ratio, i.e. not such apparent ratios as discussed in herbaceous angiosperms. Grass lignins differ from other gymnosperm and angiosperm lignins because they carry p-hyroxyphenyl units. Therefore,the SA/FA ratio in Gramineae OMTs considered to show around one. Since Gramineae is thought to be more evolved comparing to gymnosperm and dicotyledon, it is recognized that the ratio does not simply increase with the phylogenical evolution.

Although some unusual ratios were found in this experiment, it is concluded that OMTs are roughly classified into three groups, i.e. gymnosperm-, angiosperm- and grass-type OMTs, which are related to the lignin evolution.

EXPERIMENTAL

Plant Materials

The SA/FA ratios of various plants were observed in young shoots except that in Pinus, Oriza and Triticum the seedlings were used. A lot of the materials were kindly provided by the Botanical Garden of Osaka City University at Kisaichi, Osaka. Most plant materials were harvested in May. When the OMT activities were not high enough, the plant shoots were again harvested in May or June.

Extraction of OMTs

All extraction procedures are performed at 4° C. Plant materials (1 g) were homogenized in the presence of polyclar AT (0.1 g) with serum albumin (0.02 g), and 1.5 ml of 0.25 M potassium (K)-phosphate buffer (pH 7.5) containing 0.1 M each cystein and NaN_3, using an ice-cooled mortar. Then the homogenates were sqeezed with four-layered cheese-cloth and the juice obtained was centrifuged at 17000 g for 30 minutes. The supernatant obtained was used for the assay.

OMT Assay

The assay is based on the transfer of $^{14}CH_3$ groups from S-adenosyl-L-methionine to caffeate or 5-hydroxyferulate forming FA- or SA-O $^{14}CH_3$, respectively. The products formed were extracted with ether (twice, 10 ml and 5 ml). The reaction mixture contained the following components; the

enzyme solution (0.2 ml), the phenolic substrate (0.5 µmole), S-adenosyl-L-methionine-O$^{14}CH_3$ (0.05 µCi/0.5 µmole), K-phosphate (pH 7.5, 20 µmole), NaN_3 (10 µmole), $MgCl_2$ (1 µmole), cystein (10 µmole), 2-mercaptoethanol (10 µmole) and isoascorbate (10 µmole): total volume 1.3 ml. After 30, 60, 90 minutes incubation at 30° C, the enzyme reaction was terminated by the addition of 5% HCl (0.5 ml). The methylated product was extracted with ether, and the ether was evaporated under reduced pressure. The residue dissolved in 0.5 ml of dioxane was transfered in a vial with 5 ml of scintillator which contained 2,5-diphenyloxazole (4 g) and 1,4-Bis[2-(4-methyl-5-phenyloxazole-2-yl]benzene (0.1g) in toluene (1.0 l). Then, the amounts of the reaction products were calculated from the radioactivities determined with a Beckmann LS100 scintillation counter.

Mäule Color Reaction

The color test was carried out by the method of Browning.[144]

TABLE 16 SA/FA ratio in Pteridophyta and Gymnosperm OMTs

Family	Scientific Name	SA/FA	Mäule
Psilotaceae	Psilotum nudum	ND	-
Lycopodiaceae	Lycopodium clavatum	ND	-
Selaginellaceae	Selaginella tamariscina	ND	+
Equisetaceae	Equisetum arvense	ND	-
Marsileaceae	Marsilea quadrifolia	ND	-
Ginkgoaceae	Ginkgo biloba	0.1	-
Cephalotaxaceae	Cephalotaxus drupacea	ND	-
Taxaceae	Taxus cuspidata var. koraiana	0.1	-
Pinaceae	Pinus densiflora(seedlings)	0.1	-
	Pinus thunbergii(seedlings)	0.1[67]	-
	Pinus taeda(seedlings)	0.3	-
	Pinus strobus(seedlings)	0.4	-
Taxodiaceae	Sciadopitys verticillata	ND	-
	Cryptomeria japonica	ND	-
	Sequoia sempervirens	ND	-
Cupressaceae	Thuja orientalis(seedlings)	0.83[68]	-
	Thuja standishii(seedlings)	0.03[68]	-
Araucariaceae	Araucaria brasiliana	ND	-
Podocarpaceae	Podocarpus macrophylla	ND	-
Ephedraceae	Ephedra sinica	ND	+

TABLE 17 SA/FA ratio in Dicotyledon OMTs

Family	Scientific Name	SA/FA	Mäule
Trochodendraceae*	Trochodendron aralioides	1.6	+
Magnoliaceae*	Magnolia grandiflora	3.0	+
	Liriodendron tulipifera	2.5	+
Cercidiphyllaceae*	Cercidiphyllum japonicum	3.2	+
Eupteleaceae*	Euptelea polyandra	(3.6)	+
Illiciaceae*	Illicium religiosum	ND	+
Nelumbonaceae*	Nelumbo nucifera	ND	
Nymphaeaceae*	Nymphaea tetragona	ND	
	Nuphar japonicum	(2.3)	
Ranunculaceae*	Ranunculus acris	3.6	+
Paeoniaceae	Paeonia suffruticosa	ND	+
Casuarinaceae*	Casuarina sp.	ND	+
Juglandaceae*	Juglans mandshurica	ND	+
Salicaceae*	Populus euramericana	3.2[142]	+
Betulaceae*	Betula nigra	3.1	+
Fagaceae*	Quercus myrsinaefolia	2.5	+
Ulmaceae	Ulmus americana	3.2	+
Loranthaceae	Viscum album	2.2[99]	+
Leguminosae	Robinia pseudo-acacia	2.5	+
	Erythrina crista-galli	3.3[98]	-
	Glycine max(single cells)	1.9[75]	
Solanaceae	Nicotiana tabacum(single cells)	1.2[91]	
Tiliaceae	Tilia japonica	(2.3)	+
Scrophulariaceae	Paulownia tomentosa	2.9	+
Oleaceae	Forsythia suspensa	(6.4)	+
	Syringa reticulata	ND	+

TABLE 18 SA/FA ratio in Monocotyledon OMTs

Family	Scientific Name	SA/FA	Mäule
Alismataceae	*Alisma canaliculatum*	ND	
	Sagittaria trifolia	ND	
Liliaceae	*Aloë arborescens*	(1.1)	+
Juncaceae	*Juncus effusus*	ND	
Commelinaceae	*Tradescantia virginiana*	1.6	
Gramineae	*Oryza sativa*	0.9	+
	Triticum aestivum	1.0	+
	Zizania latifolia	0.97	+
	Phyllostachys pubescens	1.3[84,143]	+
Sparganiaceae	*Sparganium stoloniferum*	1.5	
Typhaceae	*Typha latifolia*	ND	+
Cyperaceae	*Eleocharis Kuroguwai*	ND	
	Scirpus triqueter	(2.2)	+

Table 16 - 18 FOOTNOTES

Young shoots were used in this experiment except the plants of scientific names which are followed by the explanatory notes. The scientific names are essentially based on reference 145 and 146.

* Polycarpiidae and Amentifloriidae, the plants of which are considered to be primitive groups in the viewpoint of polyphyletic theories in angiosperm phylogeny.[26]

ND: OMT activity was too low to evaluate the ratio SA/FA (less than 500 cpm/hr in the both FA- and SA-activities).

( ):500-1000 cpm/hr in the higher value in FA- and SA-activities.

+ or -: Positive or negative Mäule color reaction.

Suffix numbers on the SA/FA ratio: reference numbers.

FOOTNOTES (cont'd)

(Table 16)	(Table 17)	(Table 18)
matsubaran	yamaguruma	hera-omodaka
hikagenokazura	taisanboku	kuwai
iwahiba	yurinoki	aroe
sugina	katsura	i(igusa)
denjiso	husazakura	murasaki-tsuyukusa
	shikimi	ine
ichoh	hasu	komugi
chosen-maki	hitsujigusa	makomo
ichii	kohhone	mohso-chiku
akamatsu	umanoashigata	mikuri
kuromatsu	botan	gama
tehda-matsu	mokumaoh	kuro-guwai
sutorohbu-matsu	oni-gurumi	sankaku-i
kohyamaki	popura	
sugi	kaba-zoku, sp.	
sekoia	shira-kashi	
konotegashiwa	nire-zoku, sp.	
kurobe (nezuko)	yadorigi	
burajiru-matsu	nise-akashia	
inumaki	amerika-deigo	
maoh	daizu	
	tabako	
	shinanoki	
	kiri	
	rengyo	
	hashidoi	

CONCLUSIONS

CONCLUSIONS

In chapter I, a gymnosperm O-methyltransferase (OMT) was extracted from Japanese black pine (Pinus thunbergii) seedlings with differentiated primary xylem which contains lignin. The enzyme was purified 90-fold by ammonium sulfate fractionation, and by chromatography on DEAE-cellulose and Sephadex G100. The purified enzyme catalyzed caffeate-methylation to give ferulate, but scarcely did 5-hydroxyferulate to sinapate. The latter methylation was competitively inhibited by the presence of caffeate. The Vmax for caffeate was 25 times higher than 5-hydroxyferulate, and corresponding Km values were 5.11×10^{-5} and 2.77×10^{-4} M, respectively. These results indicate that syringyl nuclei are not practically formed in this conifer. The provisional molecular weight of the enzyme was estimated to be 6.7×10^{4} by gel filtration chromatography. Magnesium ions were not absolute requirement but increased enzymic activity. Thus, the OMT well explains the preponderance of guaiacyl units in the conifer lignin.

In chapter II, dicotyledonous OMTs were extracted from the differentiating xylem tissues of ten-year-old aspen (Populus euramericana) trunks. The enzymes were partially purified by ammonium sulfate precipitation, and column chromatography on DEAE-cellulose, Sephadex G200 and hydroxyapatite. The enzymes were resolved into two peaks by DEAE-cellulose

chromatography, and the molecular weights of the respective enzymes were estimated to be 72000 and 75000 by gel filtration chromatography. The enzyme corresponding to the latter peak was unstable and then the former peak enzyme was characterized. Magnesium ion showed no effect on the methylation rate. EDTA moderately stimulated the methylation rate, whereas heavy metals and SH group inhibitors strongly inhibited the enzyme activity. Km values for caffeate and 5-hydroxyferulate were estimated to be 3.8×10^{-4} and 3.1×10^{-4} M, respectively. Vmax/Km for 5-hydroxyferulate was 5.4 times greater than that for caffeate. FA- and SA-activities, which show the formation of ferulate from caffeate and the formation of sinapate from 5-hydroxyferulate respectively, were not separated during the purification and by the disc electrophoresis using polyacrylamide gel. Quercetin, cyanin and catechin were not methylated by this enzyme. The OMT in aspen trunks, where the phenolic metabolism is exclusively directed to lignin biosynthesis, catalyzes the methylation of both guaiacyl and syringyl lignin precursors, with preferential utilization of the latter substrate. These findings lead to the conclusion that the enzyme is a typical angiosperm-type OMT related to guaiacyl and syringyl lignin biosynthesis in aspen trunks. The polymorphism observed was discussed in relation to the lability of this enzyme.

In chapter III, unusual OMT was found in Mistletoe (Viscum

album). The crude enzyme apparently catalyzed only 5-hydroxyferulate-methylation, but scarecely did caffeate-methylation. However, latter methylation was recovered by purification, and the occurrence of specific inhibitor(s) was suggested in the homogenate. Thus, the OMT was also a normal angiosperm-type enzyme. The mistletoe lignin was found to be a typical angiosperm one based on the spectral (UV, IR, ^{13}C-NMR) and functional group analyses, and on degradation products (nitrobenzene oxidation and acidolysis), the analytical results of which were compared with those of the host lignin. A tracer experiment showed that L-phenylalanine was efficiently incorporated into the mistletoe lignin. Phenylalanine ammonia-lyase and cinnamate 4-hydroxylase were detected in the mistletoe by incubation of the tissue slices under illumination, and the reaction products formed were found to be identical with authentic samples by mass spectrometry. These results indicate that the mistletoe lignin is synthesized independent of its host, in other words, the mistletoe itself has an ability of lignin biosynthesis.

In chapter IV, monocotyledonous OMT was extracted from bamboo (Phyllostachys pubescens) young shoots. The enzyme was purified 97-fold by ammonium sulfate fractionation, and by chromatography on DEAE-cellulose, Sephadex G200 and G100. The purified enzyme was further analyzed by polyacrylamide gel electrophoresis and isoelectric forcusing. Thus, two

methylation, i.e. caffeate to ferulate (FA) and 5-hydroxy-ferulate to sinapate (SA), was demonstrated to be catalyzed by the same enzyme in bamboo-lignin biosynthesis. This is also supported by the ratio SA/FA which was kept constant during the purification, and by deactivation pattern on heat treatment. Methylation by bamboo OMT was found in caffeate, 5-hydroxyferulate, 3,4,5-trihydroxycinnamate, 5-hydroxyvanillin, protocatechuate, but no or few methylation in chlorogenate, iso-ferulate, m-, p-coumarate, 3,4-dihydroxyphenylacetate, 3,4-dihydroxylphenylmanderate, gallate, pyrocatechol, pyrocatechol phthalein and d-catechin. Km values for caffeate and 5-hydroxy-ferulate were 5×10^{-5} and 10^{-5}M, respectively. The former methylation, i.e. guaiacyl formation, was competitively inhibited by the latter phenolic substrate. The enzyme was an acidic protein with pI 4.61 at 4° C, showing optimal pH 8.0 with half maximal activities at pH 8.6 $\pm$ 0.2 and 6.4 $\pm$ 0.2. Possible fine control mechanism, by means of feedback inhibition and OMT-conformational change, was also discussed in relation to guaiacyl- and syringyl-lignin formation.

The OMTs in the four plant sources were purified and characterized as discribed in the chapters I - IV. In chapter V, the ratio of sinapate (SA)- to ferulate (FA)- formation (SA/FA ratio) were examined over fifty plant species of 43 families. Generally, the OMTs are found to be roughly

classified into three groups by the SA/FA ratio, i.e. gymnosperm-, angiosperm- and grass-types. OMTs in gymnosperm only catalyze FA-formation while angiosperm ones catalyze both FA- and SA-formation. Monocotyledons and herbaceous plants showed the apparent ratios inbetween typical gymnosperm and angiosperm ones. These substrate specificities well explain why gymnosperm lignins contain almost entirely guaiacyl lignins while angiosperm ones contain both guaiacyl and syringyl lignins. A few exceptional ratios were found in Cupressaceae, Trochodendron, and grass OMTs, and discussed in relation to lignin biosynthesis. The significance of the ratio for analyzing lignin evolution was also discussed.

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ACKNOWLEDGEMENTS

The author wishes to express his sincerest thanks to Dr. Takayoshi Higuchi, professor of Wood Research Institute, Kyoto University, of his guidance and encouragement during the course of this work. The author also appreciates the kind advice of Dr. Mikio Shimada and all members of Research Section of Lignin Chemisrty, Wood Research Institute, Kyoto University.

The author is grateful to Dr. Ken Shimaji, professor of Wood Research Institute, Kyoto University, and Dr. Zenzaburo Kasai, professor of Research Institute for Food Science, Kyoto University, for their suggestions and a critical reading of this manuscript.

Thanks are also due to all members of the Botanical Garden of Osaka City University, who provided many plant materials.